Identification of cholesterol-assimilating actinomycetes strain and application of statistical modeling approaches for improvement of cholesterol oxidase production by Streptomyces anulatus strain NEAE-94

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Abstract

Background Cholesterol oxidase biosensors have been applied to detect cholesterol level in different in serum and foods samples. Due to a wide range of industrial and clinical applications of microbial cholesterol oxidase, isolation and detection of new microbial source (s) of cholesterol oxidase are very important.

Results Among the potential strains, Streptomyces sp. strain NEAE-94 was chosen and identified based on cultural, morphological and physiological characteristics; in addition to the 16S rRNA sequence which had been deposited in the GenBank database under the accession number KC354803. Cholesterol oxidase production in shake flask by Streptomyces anulatus strain NEAE-94 was optimized using response surface methodology. The production parameters were first screened using a Plackett-Burman design and the parameters with significant effects on the production of cholesterol oxidase were identified. Out of the fifteen factors screened, agitation speed, cholesterol and yeast extract concentration were selected due to significant positive effects on the production of cholesterol oxidase. The optimal levels of these variables and the effects of their mutual interactions on cholesterol oxidase production were determined using Box-Behnken design. The maximum cholesterol oxidase activity (27.31 U/mL) was achieved at cholesterol concentration (4 g/L), the agitation speed (150 rpm/min) and yeast extract concentration (5 g/L). In comparison with cholesterol oxidase production by Streptomyces anulatus strain NEAE-94 before the application of Plackett-Burman design, the statistical optimization resulted in an increased production by 4.66 times.

Conclusions The maximal cholesterol oxidase activity is obtained at the following fermentation conditions: g/L (cholesterol 4, yeast extract 5, NaCl 0.5, K 2 HPO 4 1, FeSO 4 .7H 2 O 0.01, MgSO 4 .7H 2 O 0.5), pH 7, inoculum size 4% (v/v), temperature 37°C, agitation speed 150 rpm/min, medium volume 100 mL and incubation time 5 days.

Background

Cholesterol oxidase (EC 1.1.3.6) is a flavin adenine dinucleotide (FAD)-dependent enzyme that catalyzes cholesterol oxidation to cholestenone (4-cholesten-3-one) and hydrogen peroxide [1]. Cholesterol oxidase of microbial origin exhibits a wide range of industrial applications besides to its clinical applications in order to determine food and serum cholesterol levels which are important in the diagnosis of cardiovascular disease, atherosclerosis and other lipid disorders [2,3]. It plays essential role in macrophages and leukocytes lysis [4]. In addition, oxidase cholesterol also involved in the manifestation of viral (HIV) disease, bacterial disease (tuberculosis) and Alzheimer's disease [5].

In addition, cholesterol oxidase from *Streptomyces natalensis* is required for the biosynthesis of the polyene macrolide pimaricin (antifungal antibiotic) which used as mould inhibitor in the industry of food to prevent food contamination [6] and also used as an antibiotic effective in the treatment of keratitis because it interacts with the molecules of sterols present in fungal cell membranes which cause membrane disruption and leading to intracellular components leakage [7]. The microbial *Bordetella* sp. cholesterol oxidase is used as a promising therapy for lung cancer treatment (both *in vitro* and *in vivo*) and led to irreversible cell apoptosis even after addition of cholesterol [8]. *Rhodococcus equi* infects young horses as well as human immuno-compromised patients [9]. Cholesterol oxidase can be used to treat the bacterial infections of *Rhodococcus equi* for the host cells [10].

Purified bacterial cholesterol oxidase is used as a potential lethal insecticide for the boll weevil larvae "*Anthonomus grandis*" which lowers cotton yields [11]. When cholesterol oxidase is ingested with cotton boll weevil larvae, larvae die and the fertility of adult females decreases [12]. Cholesterol oxidase also exhibits insecticidal effect when ingested by pink bollworm (*Pectinophora gossypiella*), tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*) [13].

A wide range of microorganisms produce cholesterol oxidases including *Streptomyces natalensis* [6] *Streptomyces cavourensis* [14], *Mycobacterium* [9], *Arthrobacter* [15] and *Bacillus* [16] species. Pathogenic bacteria need the host cholesterol to infect the host due to its membrane damaging activity [9]. Non-pathogenic bacteria use cholesterol for growth as a carbon and energy sources. The intracellular or extracellular cholesterol oxidase produced by a variety of microorganisms.

Many scientists have sought to increase the yield of cholesterol oxidase because of low production by several microorganisms. The production of cholesterol-oxidase is greatly influenced by soluble component of the medium and culture conditions temperature, incubation time, pH, inoculum size and the rate of agitation [17], which was directly influencing cholesterol oxidase production. Response surface methodology (RSM) is an effective tool by which the optimum conditions can be determined for a multivariate system. Statistical methods were more effective than the classical method (one-variable-at-a-time) in determining the optimal concentrations of medium components used to produce cholesterol oxidase. Statistical methods offer a range of advantages, including lower experimental numbers, suitability for multi-factor experiments, search for relativity among factors, and identifying the most appropriate conditions for enzyme [18]. The implementation of statistical experimental designs include a number of steps, firstly the screening of important factors and secondly the optimization of such factors [19].

The objectives of this study were to screen cholesterol-assimilating actinomycetes strains, to identify the selected producer of cholesterol oxidase and to optimize the fermentation conditions for enhanced production of cholesterol oxidase by *Streptomyces anulatus* strain NEAE-94 using response surface methodology.

Methods

Microorganisms and cultural conditions

Streptomyces sp. strain NEAE-94 was isolated from a soil sample collected from Baltim, Kafr el-Sheikh Governorate, in the north of Egypt. *Streptomyces* sp. strain NEAE-94 isolation has been made on the plates of starch-nitrate agar medium of the following constituents (g/L): $FeSO_4.7H_2O$ 0.01; $CaCO_3$ 3; NaCl 0.5;

$MgSO_4.7H_2O$ 0.5; K_2HPO_4 1; KNO_3 2; starch 20 and agar 20 in 1L of the distilled water. The plates were incubated for seven days at 30°C. The isolate was stored as spore suspensions for further experiments in 20 percent (v/v) glycerol.

Streptomyces sp. strain NEAE-94 potentiality for cholesterol oxidase production using colony staining method

A potential of *Streptomyces* sp. strain NEAE-94 for cholesterol oxidase production was identified on the medium of an agar plate containing cholesterol as the sole source of carbon according the method of El-Naggar *et al.* [14]. It contains g/L: Cholesterol 2, potassium nitrate 2, potassium phosphate dibasic 1, magnesium sulfate heptahydrate 0.5, sodium chloride 0.5, calcium carbonate 3, Ferrous sulfate heptahydrate 0.01, agar 20 and distilled water 1L; The pH was set to 7-7.2. A colony staining procedure was applied on the growing colonies to verify cholesterol oxidase production potential. Discs of filter papers were immersed in 100 mM potassium buffer phosphate (pH 7.0) containing 0.5% cholesterol; 6% phenol; 1.7% 4-aminoantipyrine and 3 U/mL horseradish peroxidase. After that, the soaked discs were then placed on the grown colonies and incubated again for 24 hours at room temperature. Cholesterol oxidase activity was confirmed by red color development due to the formation of quinoneimine dye [20].

Inoculum preparation and cholesterol oxidase production

Streptomyces sp. strain NEAE-94 was cultivated in a 250 mL Erlenmeyer flasks containing 100 mL of broth medium, comprised of (g/L: $(NH4)_2SO_4$ 7.5; cholesterol 2; K_2HPO_4 1; $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.02; $ZnSO_4$ 0.002; $CaSO_4$ 0.002; $MnSO_4$ 0.008; NaCl 1; $CaCl_2$ 0.0002; peptone 4; yeast extract 6; starch 9; glucose 12; Tween 80 0.05) [21]. The culture of the stock was used for inoculation of broth medium by 5 disks (9 mm diameter) and was incubated for 48 h at 30°C with shaking (200 rpm/min).

100 mL of the fermentation medium was inoculated with the prepared inoculum and incubated on rotating shaker incubator at 37°C and 150 rpm/min. After the incubation time, the cell free- culture supernatant containing the crude enzyme was obtained by centrifugation of the mycelium culture using refrigerated centrifuge at 4°C and 5000 ×*g* for 30 min.

Assessment of cholesterol oxidase activity

The cholesterol oxidase activity was determined spectrophotometry by the method of El-Naggar *et al.* [22]. One enzyme activity unit (U): is the quantity of the enzyme needed for forming one µmol H_2O_2 /min at 37°C.

Cultural and morphological properties of *Streptomyces* sp. strain NEAE-94

Spore mass color, substrate mycelium color (reverse side of colony) and soluble diffusible pigments were noted on ISP media as described by Shirling and Gottlieb [23] method. The chain morphology and wall surface of spores were examined on starch-nitrate agar medium with Scanning Electron Microscopy "Jeol JSM-6360 LA operating at 20 Kv".

Physiological properties

The ability of the strain to utilize carbon sources was determined the method described by Shirling and Gottlieb [23] and determined using thirteen sterilized carbon sources namely: sucrose, ribose, D (+) glucose, L-arabinose, D (+) xylose, D (+) galactose, D (-) fructose, D (+) mannose, maltose, raffinose, rhamnose, trehalose and cellulose. Decomposition of cellulose was determined in Hutchinson liquid medium [24], cellulose was implemented in the final concentration of 1%, W/V, inoculated with the spore suspension of the strain. The strain ability to reduce nitrate to nitrite was determined using Giltay liquid medium [25]. The degradation of lecithin "lecithinase activity" was determined on plates of egg–yolk medium using Nitsch and Kützner [26] method. Spore suspension was inoculated by streak the organism on starch - nitrate agar plates and incubated for 7 days at 30°C. After that, the plates were flooded with the solution of iodine. The presence of clear zones around the growth indicated positive amylase activity [27]. Test tubes containing skimmed milk were sterilized, inoculated with spore suspension and the degree of coagulation and peptonization of milk were recorded after 7 days of incubation at 30°C [28]. Liquefaction of gelatin as a proteolytic character was determined following the method described by Preobrazhenskaya [29], as an important character for differentiation between actinomycetes. Production of melanin by *Streptomyces* sp. strain NEAE-94 was determined on slants of ISP media 1, 6 and 7; the production of brown or black soluble pigments was recorded after 7 days of incubation at 30°C that indicates positive production of melanin [23]. Degradation of casein [30], L-asparaginase production [31], sodium chloride tolerance [32], chitosanase [33] and uricase activities [34] were tested.

Molecular characterization

According to the method described by Sambrook *et al.* [35], the total genomic DNA of the strain was prepared. The PCR reaction was carried out using El-Naggar *et al.* [36] method. The amplification of 16S rRNA gene from the *Streptomyces* sp. under investigation was carried out via polymerase chain reaction (PCR) using universal primer designed to amplify the full length of the gene. The reverse primer was 1492r (5'-TACGGYTACCTTGTTACGACTT-3') and the forward primer was 27f (5'-AGAGTTTGATCMTGCCTCAG-3'). The PCR reaction was conducted in a total volume of 100 µL that contains 0.5 µL Taq polymerase, 10 µL deoxyribonucleotide 5'-triphosphate (dNTP's) (250 mM), 4 µL of both forward and reverse primers of 10 pmol, 1 µL template DNA 50 ng, 3.5 µL $MgCl_2$ (25 Mm), 10 µL PCR buffer, up to 100 µL with water.

Components of the PCR were mixed thoroughly. DNA amplification was performed using the following PCR program: Initial DNA denaturation at 94°C for 5 min; main amplification, 35 cycles each cycle consisted of: denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 2 min at 72°C followed by final extension for 10 min at 72°C. The amplified DNA was sequenced and the resulting sequence of DNA was deposited under accession number KC354803 in the GenBank NCBI database.

16S rRNA gene sequence (1536 bp) of *Streptomyces* sp. strain NEAE-94 was aligned with the 16S rRNA sequences of the related *Streptomyces* by using BLAST program [37]. The phylogenetic tree was built using version 2.1of MEGA4 software [38] via the neighbor-joining algorithm.

Selection of significant variables for on cholesterol oxidase production by Plackett–Burman design

The Plackett-Burman statistical design [39] is an efficient method used to screen and for identifying the significant variables among large number of variables that have significant effects on a process [40,41]. Plackett-Burman design was used in this study to select the medium components and environmental conditions that had a significant effect, either negatively or positively on cholesterol oxidase production out of fifteen independent variables. The Plackett-Burman experiment was conducted in 20 runs to study the effect of the selected fifteen variables on cholesterol oxidase production based on their main effects. Each variable was examined in low (–) and high (+) levels (Table 1). The chosen factors were glucose (5 and 10 g/L), starch (5 and 10 g/L), cholesterol (1 and 3 g/L), yeast extract (1 and 4 g/L), peptone (1 and 4 g/L), $(NH_4)_2SO_4$ (4 and 8 g/L), $FeSO_4.7H_2O$ (0.01 and 0.05 g/L), $MgSO_4.7H_2O$ (0.1 and 0.5 g/L), NaCl (0.5 and 1 g/L), K_2HPO_4 (0.5 and 1 g/L), pH (7 and 9), inoculum size (2 and 4%, v/v), incubation time (5 and 7 days), temperature (30 and 37●C) and agitation speed (100 and 150 rpm/min). As well, 4 dummy variables (D_1-D_4) were included.

Plackett–Burman design is based on the first order model:

Equation (3)

$$Y = \beta_0 + \sum \beta_i X_i$$

Where, Y is cholesterol oxidase production, β_0 is the model intercept, X_i is the level of each independent variable and β_i is the linear coefficient.

Optimization of cholesterol oxidase production by Box-Behnken design (BBD)

Based on the results of Plackett-Burman experiment, three factors with the highest confidence levels were selected for further optimization using Box–Behnken design [42] to estimate the optimal levels of the significant variables and to study the individual and interactions effects between the selected process variables affecting cholesterol oxidase production. These factors were agitation speed (X_1, 100–200 rpm/min), cholesterol concentration (X_2, 2–6 g/L) and yeast extract concentration (X_3, 3–7 g/L) (Table 5). Each factor is varies on three different levels (–1, 0, +1), with three center points resulting in a total of 15 different experiments (Table 5).

Linear, quadratic and interaction effects of the three significant independent variables on cholesterol oxidase production were calculated to correlate the relationship between production of cholesterol oxidase (Y) viz these factors and to predict their optimal levels using the following second order polynomial equation:

Equation (4)

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j$$

In which "Y is the cholesterol oxidase production, X_i is the coded levels of independent variables, β_0 is the regression coefficients, β_{ij} is the interaction coefficients, β_i is the linear coefficient and β_{ii} is the quadratic coefficients".

Statistical analysis

"The experimental designs and statistical analysis was performed using Design Expert software version 7 for Windows. The 3D surface plots were drawn using STATISTICA software version 8."

Results And Discussion

Actinomycete strain, *Streptomyces* sp. strain NEAE-94, has been tested for its cholesterol oxidase activity with a plate-based method, the formation of pink areas around the colonies indicated the presence of cholesterol oxidase activity (Fig. 1). The process of cholesterol-oxidation is the oxidation of cholesterol with the use of cholesterol oxidase to 4-cholesten-3-one and hydrogen peroxide. The hydrogen peroxide generated by cholesterol oxidation was then combined with 4-aminoantipyrine and phenol by peroxidase to produce quinoneimine colouration (Fig. 2). The promising strain was identified based on morphological, cultural, physiological and chemotaxonomic characteristics, in conjunction with 16S rRNA sequence.

Cultural characteristics of *Streptomyces* sp. strain NEAE-94

The isolate showed appreciable cell growth on four different media including oatmeal agar, inorganic salt-starch agar, tyrosine agar and yeast malt extract agar. Weak growth was observed on glycerol asparagines agar and peptone-yeast extract iron agar (Table 1). Aerial mycelium color was yellow-white on yeast extract-malt extract agar (Fig. 3A); yellow on oatmeal agar, tyrosine agar and inorganic salt-starch agar (Fig. 3B), while is faint yellow on peptone-yeast extract

iron agar and glycerol asparagines agar. However, the substrate mycelium develop a yellow color on oatmeal agar, inorganic salt-starch agar, yeast extract - malt extract agar; brownish orange color on tyrosine agar. Whereas, a faint orange color was developed on both glycerol asparagine agar and peptone-yeast extract iron agar. The diffusible pigment of the substrate was not pH indicator. A yellow diffusible pigment was produced in inorganic salt-starch agar, yeast extract-malt extract agar, tyrosine agar and glycerol asparagine agar; a faint yellow pigment was produced in oatmeal agar. No pigment produced in peptone-yeast extract iron agar.

Physiological properties of *Streptomyces* sp. strain NEAE-94

The physiological properties of *Streptomyces* sp. strain NEAE-94 are listed in Table 2. It utilized trehalose, D-xylose, D-mannose, rhamnose, D-galactose, raffinose, D-fructose, D-glucose, L-arabinose, sucrose, maltose and cellulose, but could not use ribose as the sole carbon source. *Streptomyces* sp. strain NEAE-94 is positive for reduction of nitrate to nitrite, protease, α–amylase (Fig. 3C), cellulase, gelatinase and asparaginase production but negative for uricase, lecithinase and chitosanase. Coagulation and peptonization of milk was positive. The strain best growing temperature was 30°C and pH of 7.0. *Streptomyces* sp. strain NEAE-94 grew in the presence of NaCl up to 5 % (w/v). It showed positive antimicrobial activities against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, but no activities were shown against *Candida albicans*, *Rhizoctonia solani*, *Aspergillus niger*, *Fusarium oxysporum*, *Alternaria solani*, *Sacchromyces cerevisiae*, *Bipolaris oryzae*, *Klebsiella pneumonia, Bacillus subtilis* and *Escherichia coli*. Melanoid pigments not formed.

Morphological features of *Streptomyces* sp. strain NEAE-94

Morphological characteristics of *Streptomyces* sp. strain NEAE-94 was observed by scanning electron micrograph after incubation on medium of starch nitrate agar at 30°C for 14 days. Microscopic observation of *Streptomyces* sp. strain NEAE-94 showed rectiflexibiles spores chains (Fig. 4). In general, chains of mature spore are long. Spore shape is rod-shaped, elongated (0.593– 0.754 x 0.995–1.341 µm), irregular and the spore surface is smooth (Fig. 4). *Streptomyces* sp. strain NEAE-94 is aerobic, mesophilic. The mycelium does not fragment.

16S rRNA gene sequence analysis and phylogenetic analysis

The obtained 16S rRNA sequence of *Streptomyces* sp. strain NEAE-94 was determined which gave an almost complete sequence with 1536 bp and further subjected to the BLAST search [37] of the GenBank database and the resultant showed homologies with other relevant sequences of many species belonging to the *Streptomyces* genus. The phylogeny of the studied isolate and closely related taxa was subjected to analysis using the program of multisequence alignment and the obtained results are illustrated in a phylogenetic tree (Fig. 5). Phylogenetic tree were constructed by using the tree-making neighbor-joining algorithm method of Saitou and Nei [43] using MEGA 3.0 software [38]. *Streptomyces* sp. strain NEAE-94 shared gene similarity of 99.38% to that of *Streptomyces anulatus* strain BZ10-24, query cover 94% (GenBank accession no. KC493992.1); 99.59% to that of *Streptomyces parvus* strain 3151, query cover 94% (GenBank accession no. EF063462.1); 99.38% to that of *Streptomyces flavofuscus* strain NRRL B-2594, query cover 94% (GenBank accession no. EF178690.1) and 99.19% to that of *Streptomyces fimicarius* strain BWL-H1, query cover 95% (GenBank accession no. MG197994.1).

Taxonomic conclusions

The whole morphological, cultural and physiological properties of *Streptomyces* sp. strain NEAE-94 and its closest phylogenetic neighbors type strains of the genus *Streptomyces* which showed significant similarities (Table 2). *Streptomyces* sp. strain NEAE-94 mainly have the same characteristics as *Streptomyces anulatus*, *Streptomyces flavofuscus*, *Streptomyces parvus* and *Streptomyces fimicarius* in that it produced rectiflexibiles spore chains and did not produce melanin pigments. However, *Streptomyces* sp. strain NEAE-94 mainly differed from *Streptomyces fimicarius* in that it generated both yellow aerial mycelium and yellow substrate mycelial pigment, where a yellow diffusible pigment on yeast extract-malt extract agar were generated. On the other hand, *Streptomyces fimicarius* lost the ability to produce a diffusible pigment and produce not distinctive substrate mycelium pigment. Also *Streptomyces* sp. strain NEAE-94 differed from *Streptomyces fimicarius* and *Streptomyces parvus* in pattern of utilization of carbon sources (Table 2). Moreover, *Streptomyces* sp. strain NEAE-94 mainly differed from *Streptomyces flavofuscus* in the diffusible pigment and color of aerial mycelium.

The comparative study between the related species of the genus *Streptomyces* and *Streptomyces* sp. strain NEAE-94 (Table 2) indicated that it mostly related to *Streptomyces anulatus* [44]. Accordingly, *Streptomyces* sp. strain NEAE-94 was identified as *Streptomyces anulatus* strain NEAE-94 (accession number is KC354803).

Screening of significant factors for production of cholesterol oxidase using Plackett–Burman design

The design matrix chosen for the testing of important factors for production of cholesterol oxidase and the appropriate responses are shown in Table 3. To investigate the effect of the chosen factors on the production of cholesterol oxidase, the experiment in 20 runs was conducted. The mycelial growth of *Streptomyces anulatus* strain NEAE-94 during cholesterol oxidase production in shake flask in submerged fermentation has been shown in Fig. 3D. The results showed broad variability of the cholesterol oxidase activity (0.87 to 11.03 U/mL) reflecting the significance of medium optimization for greater cholesterol oxidase production. The highest production of cholesterol oxidase (11.03 U/mL) was achieved in the run no. 18 using 100 mL medium/250 mL conical flask consists of (g/L): Glucose 5; starch 10; cholesterol 3; yeast extract 4; peptone 4; $(NH_4)_2SO_4$ 4; $FeSO_4.7H_2O$ 0.01; $MgSO_4.7H_2O$ 0.5; NaCl 0.5; K_2HPO_4 1 and pH 7; inoculum size was 4 % (v/v) and incubated for 5 day at 37°C using agitation speed of 150 rpm/min. In the run no. 4, the lowest production of cholesterol oxidase was obtained (0.87 U/mL) using 100 mL medium/250 mL conical flask consists of (g/L): Glucose 5; starch 10; cholesterol 1; yeast extract 1; peptone 1; $(NH_4)_2SO_4$ 4; $FeSO_4.7H_2O$ 0.05; $MgSO_4.7H_2O$ 0.1; NaCl 1; K_2HPO_4 1 and pH 9; inoculum size was 4 % (v/v) and incubated for 7 day at 37°C using agitation speed of 100 rpm/min.

Table 4 presented the statistical analysis of the responses. As regards the main effect of individual factors (Fig. 6A), 9 variables of the fifteen named temperature, agitation speed, pH, starch, cholesterol, peptone, yeast extract, ammonium sulphate and K_2HPO_4 positively affect cholesterol oxidase production,

where the remaining six factors named incubation time, glucose, inoculum size, $MgSO_4$, NaCl and $FeSO_4$ negatively affect cholesterol oxidase enzyme production. The data revealed that, the three insignificant factors are starch (G), peptone (K) and ammonium sulphate (L) with higher *P*-values and lower effects (0.09, 0.05 and 0.07; respectively) and lower contribution % (0.02, 0.01 and 0.02; respectively). Thus, backward regression was applied to remove the small effects of starch (G), peptone (K) and ammonium sulphate (L) and then the model was fitted for the significance test. The Pareto chart shows absolute effects values and illustrates the significance order of the factors that influence cholesterol oxidase production. The Pareto chart shows a reference line, any absolute effect value extending past this reference line is highly essential (Fig. 6B).

The values of the determination coefficient (R^2 = 0.9996 and the adjusted determination coefficient (Adj. R^2 = 0.9978) are very high and suggests a strong model significance [45]. The smaller *P*-value of the factor reveals that the factor is more essential for cholesterol oxidase production. Cholesterol, agitation speed with a *P*-value of <0.0001 was determined to be the most significant factors, followed by the concentration of yeast extract, incubation time (0.0001) then glucose (0.0002) (Table 4). The *F* value of the model (573.21) means that it is significant. The *P*-value < 0.05 (0.0001) implies that the model terms are significant. The first order polynomial equation representing the production of cholesterol oxidase in relation to the independent factors was obtained:

$$Y = 5.17 + 0.15\,A - 0.88\,B - 0.45\,C + 1.01\,D + 0.42\,E - 0.78\,F + 2.18\,H + 0.95\,K + 0.17\,M - 0.41\,N - 0.11\,O - 0.19\,P \quad \text{Equation (1)}$$

Where Y is the production of cholesterol oxidase and A-P are temp., time of incubation, size of inoculum, speed of agitation, pH, concentration of cholesterol, concentration of yeast extract, concentration of K_2HPO_4, NaCl, $MgSO_4$ and $FeSO_4$; respectively.

The residuals ' normal probability plot is a valuable tool for detecting and explaining the systemic deviations from the normality [46]. Fig. 6C displays a normal probability plot for the results of the experiment. The residuals have been drawn against a theoretical normal model distribution in such a manner that the points for cholesterol oxidase production should form approximately straight line. Departures from this straight line show deviations from normality. The normal probability plot of the residuals shows points close a diagonal line; so that residuals seem to be distributed nearly normal. This means the model was well designed to the findings of the experiments. Fig. 6D shows the plot of the predicted cholesterol oxidase production versus actual values, while the dots collected around a diagonal line reveals the model's excellent fit.

For the assessment of Plackett-Burman design precision, the following formula's production medium (g/L): $FeSO_4.7H_2O$ 0.01; $MgSO_4.7H_2O$ 0.5; NaCl 0.5; K_2HPO_4 1; yeast extract 4; cholesterol 3 and pH 7. The production medium was inoculated with inoculum size of 4% (v/v) and incubated at a temperature of 37°C in a shaker incubator at 100 rpm/min for 5 days. The maximum production of cholesterol oxidase of 11.03 U/mL, which was increased 2.45 times compared with the enzyme activity obtained before application of Plackett-Burman design (4.51 U/mL).

Optimization of the selected significant variables by Box–Behnken design

Depending on the statistics of the Plackett–Burman experiments results, the factors with positive impact on production of cholesterol oxidase have been maintained at a high level. While the factors with negative impact on cholesterol oxidase production (incubation time, inoculum size, $MgSO_4$, NaCl and $FeSO_4$) were fixed at their low (-1) levels. Whereas, glucose, starch, peptone and $(NH_4)_2SO_4$ have been omitted in the subsequent experiments.

Box–Behnken design [42] was used to obtain the optimal levels of the most significant factors influencing cholesterol oxidase production by *Streptomyces anulatus* strain NEAE-94 and to study the interaction effects between these factors. In the current study, fifteen experiments with various combinations of agitation speed, cholesterol concentration and yeast extract concentration were performed and the experimental and predicted cholesterol oxidase production and residuals for the fifteen trials are provided in Table 5.

Based on the variations in the agitation speed, cholesterol concentration and yeast extract concentration; the results show variations in the cholesterol oxidase production. Cholesterol oxidase production ranged from 5.64-27.31 U/mL. The lowest cholesterol oxidase production by *Streptomyces anulatus* strain NEAE-94 (5.64 U/mL) was achieved in the 13th run when agitation speed was 100 rpm/min, cholesterol concentration was 4 g/L and yeast extract concentration was 3 g/L. The maximum value of cholesterol oxidase production was achieved in the 12th run with value of 27.31 U/mL, when agitation speed was 150 rpm/min, cholesterol concentration was 4 g/L and yeast extract concentration was 5 g/L.

The maximum cholesterol oxidase production obtained from this research (27.31 U/mL) by *Streptomyces anulatus* strain NEAE-94 is superior and greater than most of the other reported values such as the cholesterol oxidase production by *Rhodococcus equi* no. 23 (0.24 U/mL) [47], *Micrococcus* sp. (3.68 U/mL) [48], *Streptomyces* A (2.44 U/mL) [49], *Brevibacterium* sp. (1.483 U/mL) [50], *Streptomyces lavendulae* (2.21 U/mL) [51], *Streptomyces* sp. (6.2 U/mL) [52], *Bacillus cereus* (1.67 U/mL) [53] and *Streptomyces fradiae* (0·03 U/mL) [21].

The analysis of variance (ANOVA) for multiple regression analysis

Tables 6 contain multiple regression analysis and ANOVA for the results of the Box–Behnken design model. The ANOVA of the multiple regression analysis show the model to be highly significant, as can be seen from the low probability value (<0.0001) and the value of Fisher's *F*-test (113.82) (Table 6). The current R^2 and adjusted R^2 values were 0.9951 and 0.9864; respectively, implies that the model is appropriate to represent the real relationship between cholesterol oxidase production and the selected factors. The largest R^2 value showed that experimental and expected cholesterol oxidase production values are in excellent agreement [54]. Predicted R^2 value of 0.9427 shows that the model is sufficient to predict the value of the production of cholesterol oxidase in the range of factors used. Accuracy and reliability of the model can be seen in the small percentage of the coefficient of variation value (CV=5.44%), mean value (15.16), adequate precision value (31.469), PRESS value (40.03) and standard deviation value (0.82) (Table 6).

The significance of each coefficient was defined in terms of both *P* and *F* values listed in Table 6. It can be seen from the *P*-values and *F*-values that the linear coefficients of cholesterol concentration, interaction among agitation speed and cholesterol concentration; agitation speed and yeast extract concentration; cholesterol concentration and yeast extract concentration and quadratic effects of agitation speed, cholesterol concentration and yeast extract concentration are significant as it is evident from the *F*-values of 119.05, 7.80, 62.24, 7.80, 321.59, 79.45, 526.73; respectively, and *P*-values of 0.0001, 0.0383, 0.0005, 0.0383, <0.0001, 0.0003, <0.0001; respectively. On the other hand, *P*-values of the linear coefficients of agitation speed (X_1) and yeast extract concentration (X_3) indicate that they had a non significant effect on cholesterol oxidase production by the strain under study.

A high quadratic model fitting with Box–Behnken design used for cholesterol oxidase production by *Streptomyces anulatus* strain NEAE-94, with a non-significant lack of fit (*F*-value 1.51 and *P*-value = 0.4219) and a very low *P*-value< 0.0001 was shown by the fit summary results (Table 7). The largest adjusted and predicted R^2 of 0.9864 and 0.9427 and the lowest standard deviation (0.82) was reported in the summary statistics of the quadratic model.

The non- significant lack-of-fit, the high value of adjusted and predicted R-squared, low PRESS value, high *F*-value, low standard deviation and high adequate precision indicates the validity and high degree of accuracy of the model prediction for production of cholesterol oxidase by *Streptomyces anulatus* strain NEAE-94.

The optimum levels of agitation speed, cholesterol concentration and yeast extract concentration giving the maximum cholesterol oxidase production was evaluated by a second-order polynomial equation. Cholesterol oxidase production can be predicted by applying the following second-order regression equation in terms of the independent variables:

$$Y = 26.55+0.04X_1+3.18X_2+0.35X_3+1.15X_1X_2-3.25X_1X_3+1.15X_2X_3-7.69X_1^2-3.82X_2^2-9.84X_3^2 \quad \text{Equation (2)}$$

Where Y is the cholesterol oxidase production, X_1 is the coded value of agitation speed, X_2 is the coded value of cholesterol concentration and X_3 is the coded value of yeast extract concentration.

Three dimensional (3D) surface and Contour plots

To understand the interaction among the three factors (X_1 - X_3) and the optimum values of each factor required for the maximum cholesterol oxidase production, the 3D curves and contour plots were plotted by plotting the cholesterol oxidase production on the Z axis versus two factors are allowed to vary and the third variable is fixed at its zero level (shown in Fig. 7A–C). Fig 7A represents the cholesterol oxidase production as the simultaneous effect of agitation speed (X_1), cholesterol concentration (X_2) while yeast extract was kept at the central point (5 g/L). The cholesterol oxidase activity increased with the rise in the cholesterol concentration at middle level of agitation speed. Further rise in the concentration of cholesterol or of agitation speed leads however to a gradually reduction in cholesterol oxidase activity. It could be seen from Fig. 7A that the greater agitation speed support comparatively small cholesterol oxidase production. By solving the equation (2), the highest cholesterol oxidase production of 27.21 U/mL could be reached using 5 g/L yeast extract at the optimal predicted levels of agitation speed and cholesterol concentration of 150 rpm/min and 4.8 g/L; respectively.

Enhanced production of cholesterol oxidase was recorded with the use of different compounds as cholesterol, yeast extract [21]; malt extract, potato starch and peptone [55] as substrates. Yehia *et al*. [56] noted that the growth and breakdown of cholesterol by the tested bacterial isolates were largely and severely affected by cholesterol concentration in the cultural medium. The largest cholesterol breakdown by the *Enterococcus hirae* was achieved using 1 g/L cholesterol. Sojo *et al*. [57] and Yazdi *et al*. [21] reported that the largest cholesterol breakdown and maximum cholesterol oxidase production by the *Rhodococcus erythropolis* and *Streptomyces fradiae* was achieved using 2 g/L cholesterol.

There are different and opposite effects of nitrogen sources on the production of cholesterol oxidase in the literature. Voelker and Altaba [58] recorded an increase in the production of cholesterol oxidase by organic nitrogen much greater than inorganic nitrogen. The reason is that organic nitrogen may contain the most types of growth factors and amino acids required for the microbial growth and could be immediately metabolized through cells, thereby supporting the cholesterol oxidase production [51].

Moradpour *et al*. [59] and Ahmad [60] reported that the best sources of nitrogen for cholesterol decomposition by *Pseudonocardia compacta* S-39 were ammonium nitrate, ammonium sulphate and sodium nitrate [61]. It has been found that an organic source of nitrogen has been found to have more influence on the activity of cholesterol oxidase than the inorganic source of nitrogen. Maximum cholesterol oxidase production was recorded with 0.3 %, w/v yeast extract [21] and 0.4–0.5 %, w/v [47] as the best source of nitrogen by *Rhodococcus equi* 2C and by *Rhodococcus equi* no. 23; respectively. On the other hand, Liu *et al*. [62] reported that the best sources of nitrogen for maximum cholesterol oxidase production by *Arthrobacter simplex* were ammonium salts.

Appropriate oxygen must be given in the fermentation media using shaken cultures to satisfy the organism's growing demands and to produce the desired end product. The suitable agitation speed ensures that the dissolved oxygen in the medium is adequately supplied and can become vital for microbial biosynthesis of certain end products.

The three-dimensional surface and contour plots in Fig. 7B illustrates cholesterol oxidase production as function of agitation speed (X_1) and yeast extract concentration (X_3) while cholesterol concentration (X_2) was fixed at the central point (4 g/L). Fig. 7B indicates that low cholesterol oxidase production is supported by lower and higher agitation speed (X_1) levels, an increase in agitation speed, the cholesterol oxidase production increases beyond 150 rpm/min after which cholesterol oxidase production was reduced. Lower and high concentrations of yeast extract (X_3) results in low cholesterol oxidase production, and the maximum cholesterol oxidase production, obviously obtained at the central level of the yeast extract concentration. By analysis of Fig. 7B and solving the equation (2), the maximum predicted cholesterol oxidase production of 26.55 U/mL could be reached using 4 g/L cholesterol at the optimal predicted levels of agitation speed (150 rpm/min) and yeast extract concentration (5 g/L).

Fig 7C shows cholesterol oxidase production as influenced by cholesterol concentration (X_2) and the concentration of yeast extract (X_3) by maintaining the agitation speed at the central point (150 rpm/min). With an increased concentrations of both cholesterol and yeast extract; cholesterol oxidase production by the selected strain (*Streptomyces anulatus* strain NEAE-94) was improved and the maximum cholesterol oxidase production has been obtained at the middle levels of two factors, and further increase of cholesterol concentration or yeast extract concentration decrease cholesterol oxidase activity. By analysis of Fig. 7C and solving the equation (2), the maximal predicted cholesterol oxidase production of 27.21 U/mL could be reached using agitation speed (150 rpm/min) and the optimal predicted levels of 4.8 g/L cholesterol and yeast extract concentration (5 g/L).

In this study, the maximum cholesterol oxidase production (27.31 U/mL) is obtained through the following medium formula (g/L): yeast extract 5, cholesterol 4, $FeSO_4.7H_2O$ 0.01, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, K_2HPO_4 1, pH 7, inoculum size 4 % (v/v), temperature 37°C, agitation speed 150 rpm/min, medium volume 100 mL and incubation time 5 days.

El-Naggar *et al*. [14] used the 2-level Plackett–Burman experimental design in 20 experimental run to evaluate the importance of fifteen process variables of medium components and operating conditions for the production of cholesterol oxidase by *Streptomyces cavourensis* strain NEAE-42. The most important factors that significantly influenced cholesterol oxidase production were initial pH, cholesterol and $(NH_4)_2SO_4$ concentrations. El-Naggar *et al*. [14] reported that the optimal levels of the three selected process factors for maximum cholesterol oxidase production (20.521 U/mL) as obtained from central composite design were pH 8; cholesterol concentration 3 g/L; $(NH_4)_2SO_4$ 8 g/L. However, Ahmad and Goswami [63] optimized the medium for production of cholesterol oxidase by *Rhodococcus* sp. NCIM 2891 using the classical and statistical methods. They reported that the maximum cholesterol oxidase production (3.25 U/mL) was obtained in the statistically optimized medium under the optimal levels of the process factors that were 2.5g/L $(NH_4)_2HPO_4$, 9 g/L yeast extract and 3.5 g/L cholesterol in approximately 60 hours of cultivation at 30°C and pH 7.0. On the other hand, Srivastava *et al*. [64] apply various statistical optimization techniques to enhance the cholesterol oxidase production by *Streptomyces rimosus* MTCC 10792. They reported that, out of the examined factors, yeast extract, dextrose, starch and ammonium carbonate were the most significant factors and the maximum cholesterol oxidase production was 5.41 U/mL in the optimized medium using the optimum concentrations of the four variables that were (g/100 mL medium): 0.05 ammonium carbonate, 0.1 starch, 0.8 dextrose and 0.99 yeast extract. Moradpour *et al*. [59] screened various process variables that had a major impact on cholesterol oxidase production by *Streptomyces badius* using Plackett-Burman design and optimized these variables using Box-Behnken design. They reported that, yeast extract, pH, Tween 20 and temperature were the most significant factors and the maximum cholesterol oxidase production by *Streptomyces badius* was 2.05 U/mL in the optimized medium using the optimum levels of the four variables that were determined to be: yeast extract, 0.45 %; pH, 6.5; Tween 20, 0.05 % and 30°C. Moreover, ElBaz *et al*. [65] used a two-step statistical approach to optimize the production of cholesterol oxidase from *Bacillus pumilus*. The maximum cholesterol oxidase production (90 U/mL) was obtained after 6 days of fermentation at pH 8 with medium/flask ratio of 0.35 and the concentrations of cholesterol, NH_4NO_3, yeast extract and Tween 80 were 0.2, 0.3, 1 and 0.2%; respectively.

Kuppusamy and Kumar [53] used traditional one variable at a time method to find the key nutritional components such as different carbon sources, nitrogen sources, metal ions and different physical parameters like incubation time, temperature and pH in order to enhance cholesterol oxidase production by *Bacillus cereus* strain KAVK4. The highest production of cholesterol oxidase was achieved under flask conditions using the optimal levels of fructose (as carbon source) in the production medium at a concentration of 2%, ammonium nitrate (as nitrogen source) at a concentration of 0.2% and magnesium sulphate as metal ion source at a concentration of 0.03%. The maximum production of cholesterol oxidase by *Bacillus cereus* strain KAVK4 of 1.67 U/mL was achieved at the optimum process variable values (incubation time was at 32 hrs, pH 7.5 at room temperature).

Yang and Zhang [50] used a three level central composite design to investigate the correlation between three independent variables (cholesterol, Tween-80 and treatment time) and cholesterol oxidase production by *Brevibacterium* sp. They found that, the optimal values of the three independent variables resulted in highest cholesterol oxidase production by *Brevibacterium* sp. (1.483 U/mL) were determined to be: 22.361 (min) treatment time, 0.2932% (v/v) Tween-80, 4.076 g/L cholesterol. As well, El-Naggar *et al*. [22] used the Plackett-Burman design to evaluate the influence of nutritional and environmental variables for cholesterol oxidase production by *Streptomyces aegyptia* strain NEAE-102. They found that, out of fifteen variables screened by Plackett-Burman design experiments, pH, incubation time and cholesterol concentration were the most significant variables for cholesterol oxidase production. El-Naggar *et al*. [22] used a face centered central composite design to optimize the levels and analyze the combined effects of pH, incubation time and cholesterol concentration. The optimum levels of these variables for the highest cholesterol oxidase production (15.631 U/mL) were determined to be: pH 6, 5 days of incubation time and 3 g/L cholesterol.

Varma and Nene [55] studied cholesterol oxidase production by *Streptomyces lavendulae* NCIM 2421. A peak of cholesterol oxidase activity of 1.8 U/mL was detected in 72 h. They found that, *Streptomyces lavendulae* NCIM 2421 is a constitutive producer of cholesterol oxidase where the addition of cholesterol to the medium did not enhance cholesterol oxidase activity. Whereas, Chauhan *et al*. [51] used orthogonal array method and response surface methodology to optimize medium for cholesterol oxidase production by *Streptomyces lavendulae* NCIM 2499. They reported that the model predicted maximum cholesterol oxidase production (2.21 U/mL) could be achieved after 72 h of incubation using the medium of the following composition (g/L): sodium chloride 0.7, $MgSO_4$ 2, K_2HPO_4 0.6, soyabean meal 20, malt extract 20 and glycerol 10 mL/L. An initial pH of 7.5 supported the maximum production of cholesterol oxidase. Moreover, Srivastava *et al*. [64] standardized the process of cholesterol oxidase production by studying different range of various parameters at shake flask level. They found that the maximum cholesterol oxidase production by *Streptomyces rimosus* was achieved using the optimal levels of process variables which found to be inoculum size (3%, v/v), pH (7), incubation temperature (30°C), incubation time (48 h) and agitation speed (200 rpm/min). Moving above or below from the optimum range of these parameters, decrease in enzyme activity was observed. Fazaeli *et al*. [66] achieved maximum cholesterol oxidase production by *Escherichia coli* when the induced culture production medium was incubated for 24 hours at 15°C. Also, Niwas *et al*. [52] studied the effect of process variables on cholesterol oxidase production by *Streptomyces* sp. at shake flask level. The results indicated that the maximum cholesterol oxidase production (6.2 U/mL) was achieved using 0.05%, w/v cholesterol, pH 7 and 35°C, 200 rpm/min.

Verification Of The Model

The maximum cholesterol oxidase production by *Streptomyces anulatus* strain NEAE-94 as obtained using Box–Behnken design were verified experimentally and compared with the predicted cholesterol oxidase production. The maximum experimental cholesterol oxidase production was 27.31 U/mL obtained at the optimal levels of the process variables: agitation speed (150 rpm/min), cholesterol concentration (4 g/L) and yeast extract concentration (5 g/L). Using the optimal levels of the process variables, the experimental cholesterol oxidase production was verified and compared with the predicted value cholesterol oxidase production (26.55 U/mL). The verification revealed a high degree of model accuracy (97.21%).

Conclusion

In the present study, the potential of *Streptomyces anulatus* strain NEAE-94 for cholesterol oxidase production has been investigated. *Streptomyces anulatus* strain NEAE-94 could be used as a promising, efficient source for cholesterol oxidase production. The maximum cholesterol oxidase production after statistical optimization of of fermentation process variables was 27.31 U/mL with a fold of increase 6.06 compared with the cholesterol oxidase production before applying Plackett-Burman design (4.51 U/mL).

Declarations

Competing interests

The authors declare no competing financial and/or non-financial interests.

Authors' contributions

"NEE proposed the research concept, designed the experiments, providing necessary tools for experiments, experimental instructions, performed the statistical analysis, analyzed and interpreted the data and wrote the manuscript. NME carried out the experiments, participated in writing of the manuscript. All authors read and approved the final manuscript."

Ethics approval and consent to participate

Not applicable

Consent for publication

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Tables

Table 1. Cultural properties of *Streptomyces* sp. strain NEAE-94

Medium	Color of			Growth
	Diffusible pigment	Substrate mycelium	Aerial mycelium	
ISP 2	Yellow	Yellow	Yellow-white	Excellent
ISP 3	Faint yellow	Yellow	Yellow	Excellent
ISP 4	Yellow	Yellow	Yellow	Excellent
ISP 5	Yellow	Faint orange	Faint yellow	Weak
ISP 6	Non-pigmented	Faint orange	Faint yellow	Weak
ISP 7	Yellow	Brownish orange	Yellow	Very good

Table 2. Phenotypic properties of *Streptomyces* sp. strain NEAE-94 and related *Streptomyces* species. Reference species properties have been taken from Bergey's Manual of Systematic Bacteriology [44].

Characteristic	*Streptomyces* sp. strain NEAE-94	*S. anulatus*	*S. flavofuscus*	*S.fimicarius*	*S. parvus*
Aerial mycelium on ISP medium 2	Yellow-whitish	Yellow or white	Yellow, green-yellow	Yellow or white	Yellow
Diffusible pigment	Yellow	Trace of yellow	Yellow-gray-brown		Yellow
Substrate mycelium on ISP medium 2	Yellow	Pale yellow; grayish yellow or yellowish brown	Yellow- gray-brown	Not distinctive	Not distinctive
Surface of spores	Smooth	Smooth	Smooth	Smooth	Smooth
Morphology of spore chain	*Rectiflexibles*	*Rectiflexibiles*	*Rectiflexibiles*	*Rectiflexibiles*	*Rectiflexibiles*
Melanin production on Tyrosine agar, peptone-yeast extract iron agar or tryptone-yeast extract broth	–	–	–	–	–
Utilization of carbon sources					
D(+) glucose, D(-) fructose, L-arabinose, D(+) xylose, rhamnose	+	+		+	+
Sucrose, raffinose	+	+		±	±
D(+) mannose, cellulose	+	+		+	
Trehalose, D(+) galactose, maltose	+				
Ribose	–				

Abbreviations: "+, Positive; –, Negative; ±, Doubtful; Blank cells, no data available. L-asparaginase, gelatinase, cellulose, protease and α–amylase were produced by *Streptomyces* sp. strain NEAE-94 while chitosanase, lecithinase and uricase were not produced. Maximum NaCl tolerance (5%, w/v).The optimal growth temperature was 30ºC and optimal pH was 7.0. It exhibited positive antimicrobial activities against *Pseudomonas aeruginosa, Staphylococcus aureus*. Nitrate reduction, coagulation and peptonization of milk were positive".

able 3. Twenty-trial Plackett–Burman experimental design used for cholesterol oxidase production.

Std	Run no.	Coded levels of independent variables											
		Temperature (ºC)	Incubation time (Days)	Inoculum size % (v/v)	Agitation speed (rpm/min)	pH	Glucose (g/L)	Starch (g/L)	Cholesterol (g/L)	Peptone (g/L)	Yeast extract (g/L)	$(NH_4)_2SO_4$(g/L)	K_2HPO_4(g
6	1	-1	-1	1	1	-1	1	1	-1	-1	1	1	
12	2	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	
4	3	1	1	-1	1	1	-1	-1	1	1	1	1	
15	4	1	1	1	-1	1	-1	1	-1	-1	-1	-1	
20	5	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	
7	6	-1	-1	-1	1	1	-1	1	1	-1	-1	1	
2	7	-1	1	1	-1	-1	1	1	1	1	-1	1	
18	8	-1	-1	1	1	1	1	-1	1	-1	1	-1	
16	9	1	1	1	1	-1	1	-1	1	-1	-1	-1	
10	10	-1	1	-1	-1	-1	-1	1	1	-1	1	1	
14	11	1	1	-1	1	-1	1	-1	-1	-1	-1	1	
1	12	1	1	-1	-1	1	1	1	1	-1	1	-1	
17	13	-1	1	1	1	1	-1	1	-1	1	-1	-1	
8	14	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	
11	15	1	-1	1	-1	-1	-1	-1	1	1	-1	1	
5	16	-1	1	1	-1	1	1	-1	-1	1	1	1	
19	17	1	-1	-1	1	1	1	1	-1	1	-1	1	
3	18	1	-1	1	1	-1	-1	1	1	1	1	-1	
13	19	1	-1	1	-1	1	-1	-1	-1	-1	1	1	
9	20	1	-1	-1	-1	-1	1	1	-1	1	1	-1	
Variable level		A	B	C	D	E	F	G	H	J	K	L	M
-1		30	5	2	100	7	5	5	1	1	1	4	0.5
1		37	7	4	150	9	10	10	3	4	4	8	1.0

Table 4. Statistical analysis of Plackett-Burman design

Term	Coefficient	Effect	% Contribution	*F*-value	*P*-value Prob > *F*
Intercept	5.17			573.21	0.0001*
A-Temperature	0.15	0.30	0.25	17.15	0.0256*
B-Incubation time	-0.88	-1.77	8.86	615.20	0.0001*
C-Inoculum size	-0.45	-0.91	2.33	161.94	0.0010*
D-Agitation speed	1.01	2.02	11.58	804.04	< 0.0001*
E-pH	0.42	0.84	2.00	138.84	0.0013*
F-Glucose	-0.78	-1.56	6.92	480.30	0.0002*
G-Starch	0.04	0.09	0.02	9.87×10^{-3}	0.9271*
H-Cholesterol	2.18	4.37	54.13	3757.27	< 0.0001*
J-Peptone	0.02	0.05	0.01	3.38×10^{-3}	0.9573
K-Yeast extract	0.95	1.90	10.20	707.66	0.0001*
L-$(NH_4)_2SO_4$	0.04	0.07	0.02	7.37×10^{-3}	0.9370
M- K_2HPO_4	0.17	0.34	0.33	22.89	0.0174*
N-NaCl	-0.41	-0.83	1.95	135.29	0.0014*
O- $MgSO_4.7H_2O$	-0.11	-0.23	0.14	9.98	0.0509
P- $FeSO_4.7H_2O$	-0.19	-0.38	0.40	27.95	0.0132*
PRESS	12.92	Adeq Precision		79.34	
C.V. %	3.08	Pred R^2		0.9261	
Mean	5.17	Adj R^2		0.9978	
Std. Dev.	0.16	R^2		0.9996	

* Significant values, "*F*: Fishers's function, PRESS is the predicted residual sum of squares, C.V. % is the coefficient of variation%"

Table 5. Three-level Box-Behnken design representing cholesterol oxidase production as influenced by agitation speed (X_1), cholesterol concentration (X_2) and yeast extract concentration (X_3).

Std	Run	Agitation speed		Cholesterol concentration		Yeast extract concentration		Cholesterol oxidase activity (U/mL)		Residuals
		Coded	Actual (rpm/min)	Coded	Actual (g/L)	Coded	Actual (g/L)	Experimental	Predicted	
15	1	0	150	0	4	0	5	26.44	26.55	-0.10
13	2	0	150	0	4	0	5	25.88	26.55	-0.66
1	3	-1	100	-1	2	0	5	12.90	12.97	-0.07
9	4	0	150	-1	2	-1	3	10.31	10.50	-0.19
2	5	1	200	-1	2	0	5	11.48	10.74	0.74
7	6	-1	100	0	4	1	7	13.13	12.58	0.55
3	7	-1	100	1	6	0	5	16.29	17.03	-0.74
12	8	0	150	1	6	1	7	17.76	17.57	0.19
11	9	0	150	-1	2	1	7	8.44	8.91	-0.47
10	10	0	150	1	6	-1	3	15.03	14.56	0.47
4	11	1	200	1	6	0	5	19.47	19.40	0.07
14	12	0	150	0	4	0	5	27.31	26.55	0.77
5	13	-1	100	0	4	-1	3	5.64	5.38	0.27
6	14	1	200	0	4	-1	3	11.40	11.95	-0.55
8	15	1	200	0	4	1	7	5.89	6.16	-0.27

Table 6. Regression statistics and analysis of variance (ANOVA) for Box-Behnken design results.

Source	*df*	Coefficient estimate	*F*-value	*P*-value *Prob >F*
Model	9	26.55	113.82	< 0.0001*
X_1	1	0.04	0.02	0.9071
X_2	1	3.18	119.05	0.0001*
X_3	1	0.35	1.48	0.2782
$X_1 X_2$	1	1.15	7.80	0.0383*
$X_1 X_3$	1	-3.25	62.24	0.0005*
$X_2 X_3$	1	1.15	7.80	0.0383*
X_1^2	1	-7.69	321.59	< 0.0001*
X_2^2	1	-3.82	79.45	0.0003*
X_3^2	1	-9.84	526.73	< 0.0001*
R^2	0.9951	Std. Dev.	0.82	
Adj R^2	0.9864	Mean	15.16	
Pred R^2	0.9427	C.V. %	5.44	
Adeq Precision	31.4690	PRESS	40.03	

* Significant values, "*df* : Degree of freedom, *F*: Fishers's function, *P*: Level of significance, C.V: Coefficient of variation".

Table 7. Fit summary for Box-Behnken design results

Sequential Model Sum of Squares					
Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	***P*-value** ***Prob >F***
Linear vs Mean	81.84	3	27.28	0.49	0.6986
2FI vs Linear	52.84	3	17.61	0.25	0.8594
Quadratic vs 2FI	560.74	3	186.91	275.33	< 0.0001*
Residual	1.04	2	0.52		
Lack of Fit Tests					
Source	**Sum of Squares**	***df***	**Mean Square**	***F*-value**	***P*-value** ***Prob >F***
Linear	615.94	9	68.44	131.78	0.0076*
2FI	563.10	6	93.85	180.71	0.0055*
Quadratic	2.36	3	0.79	1.51	0.4219
Pure Error	1.04	2	0.52		

Model Summary Statistics					
Source	**Standard deviation**	**R-Squared**	**Adjusted R-Squared**	**Predicted R-Squared**	**PRESS**
Linear	7.49	0.1171	-0.1237	-0.3361	933.70
2FI	8.40	0.1927	-0.4127	-0.9671	1374.66
Quadratic	0.82	0.9951	0.9864	0.9427	40.03

* Significant values, "*df* : degree of freedom, PRESS: sum of squares of prediction error, two factors interaction: 2FI"

Figures

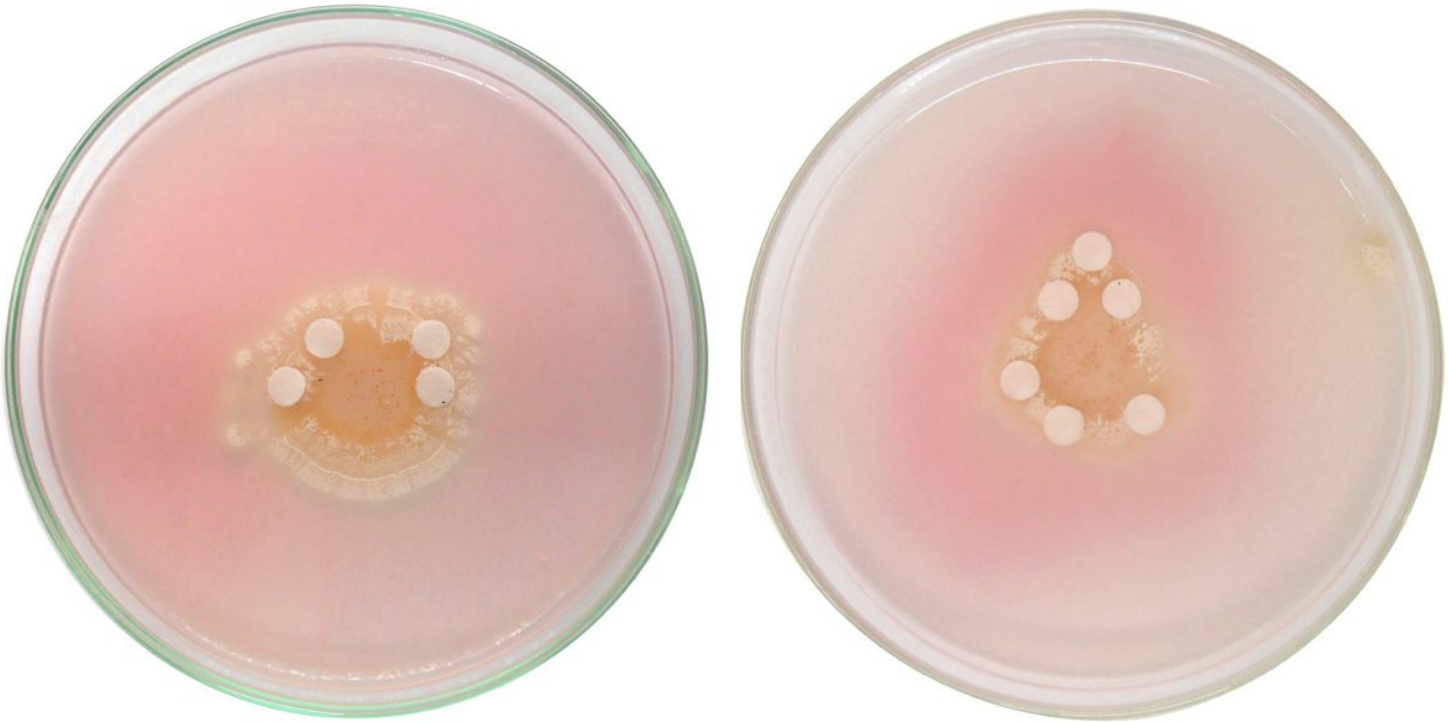

Figure 1

Cholesterol oxidase production of Streptomyces sp. NEAE-94 detected by plate assay method.

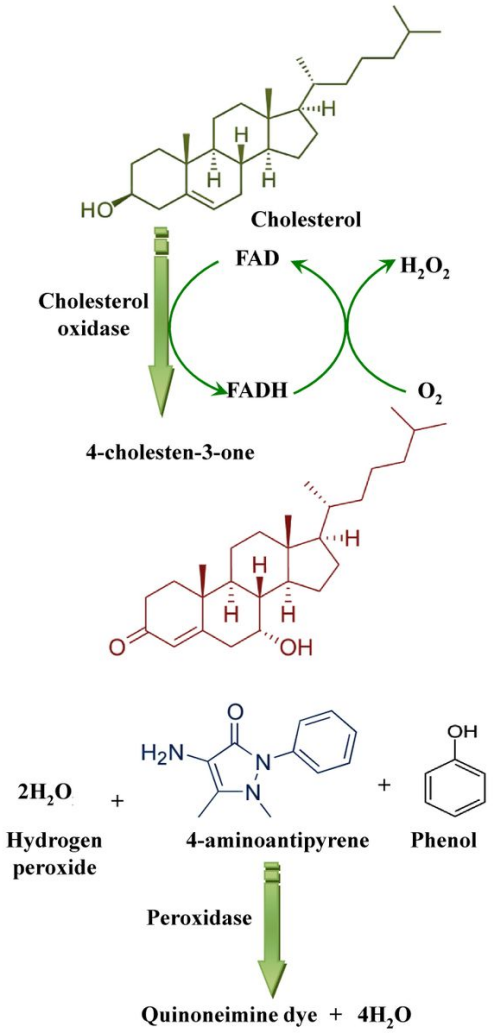

Figure 2

Mechanism of reaction catalyzed by cholesterol oxidase.

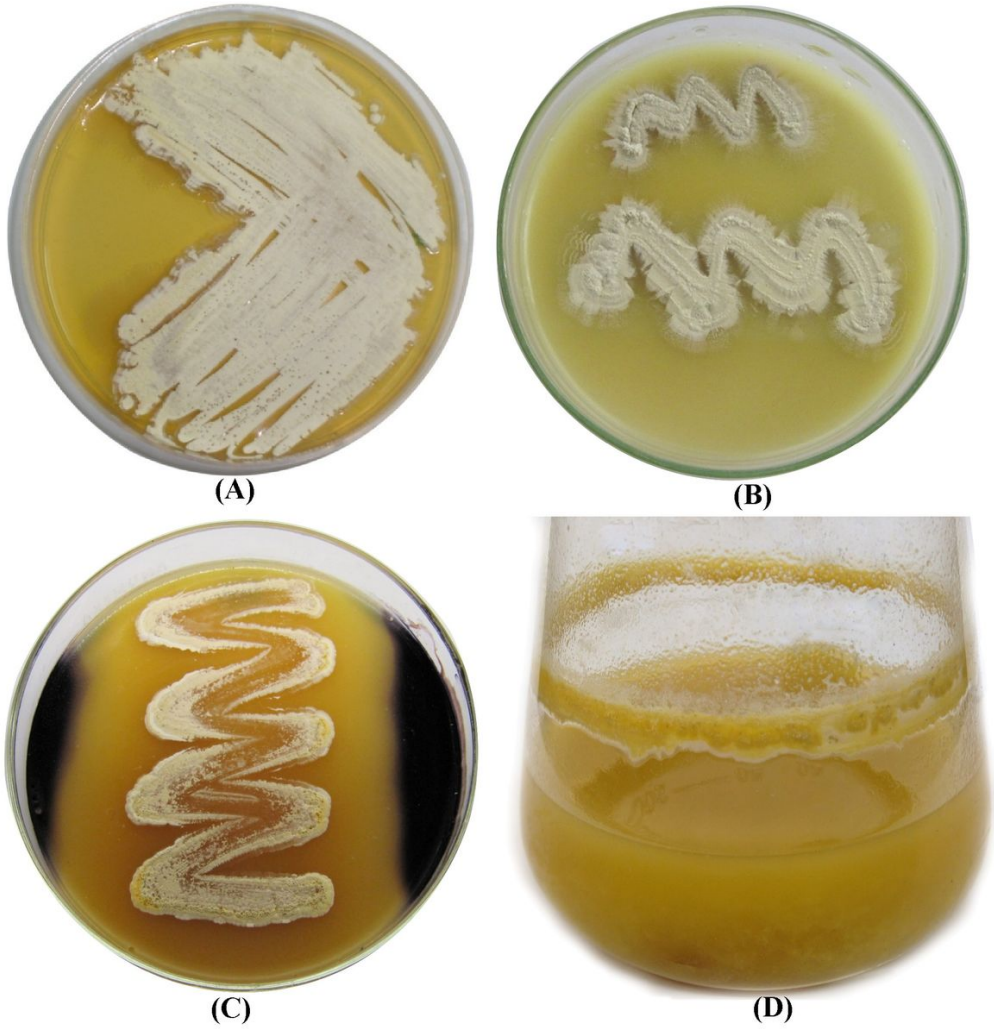

Figure 3

The colored photograph of Streptomyces sp. NEAE-94 aerial mycelium after growth on yeast extract -malt extract agar (A) and inorganic salt-starch agar media (B) at 30°C for 7 days, (C) plate assay showing starch hydrolysis by Streptomyces sp. strain NEAE-94 and (D) cholesterol oxidase production in submerged fermentation.

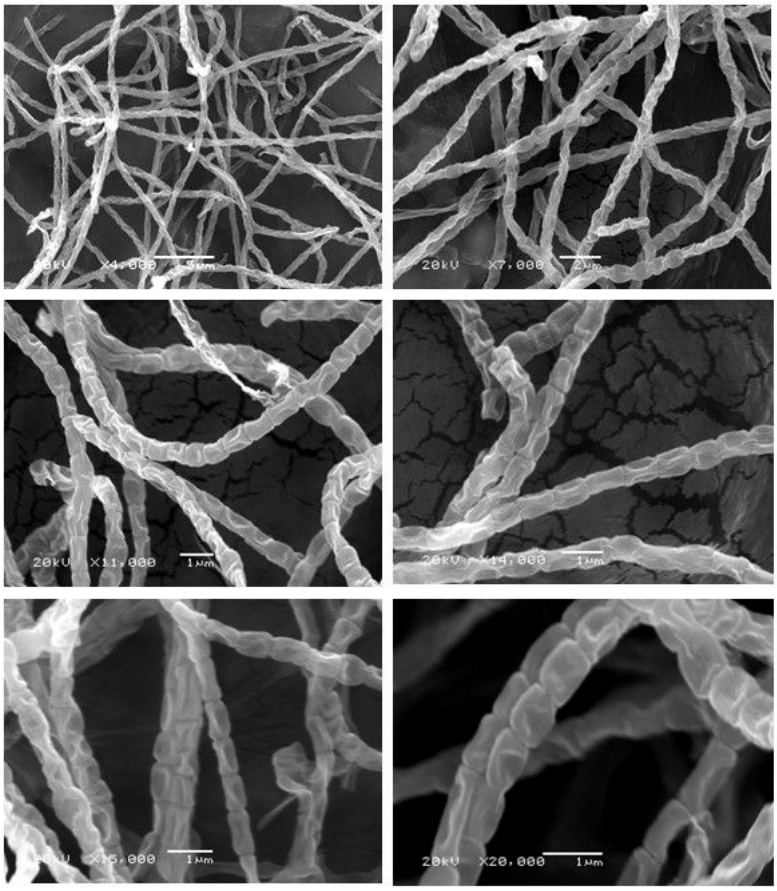

Figure 4

Spore-chain and spore-surface morphology of Streptomyces sp. strain NEAE-94 under scanning electron microscope at magnification of 4000X-20000X.

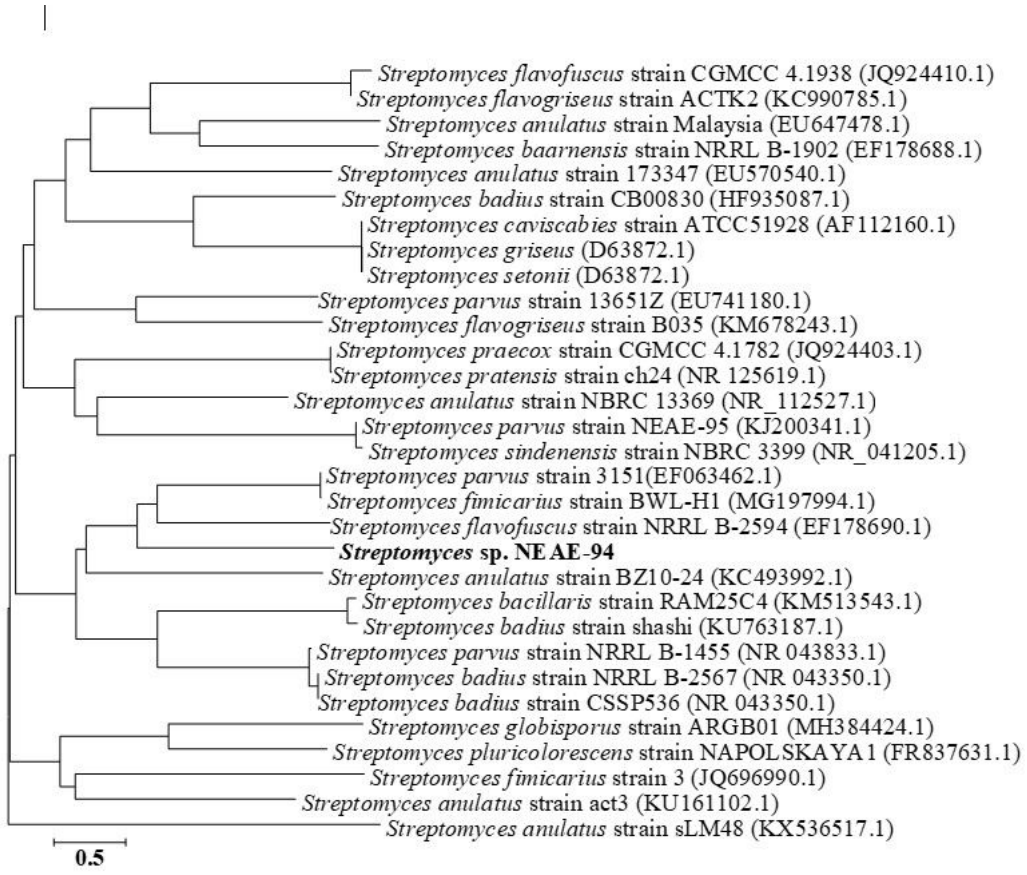

Figure 5

Phylogenetic tree obtained by Neighbour-joining method showing the relationship between Streptomyces sp. strain NEAE-94 and other related species of Streptomyces based on the 16S rRNA sequences.

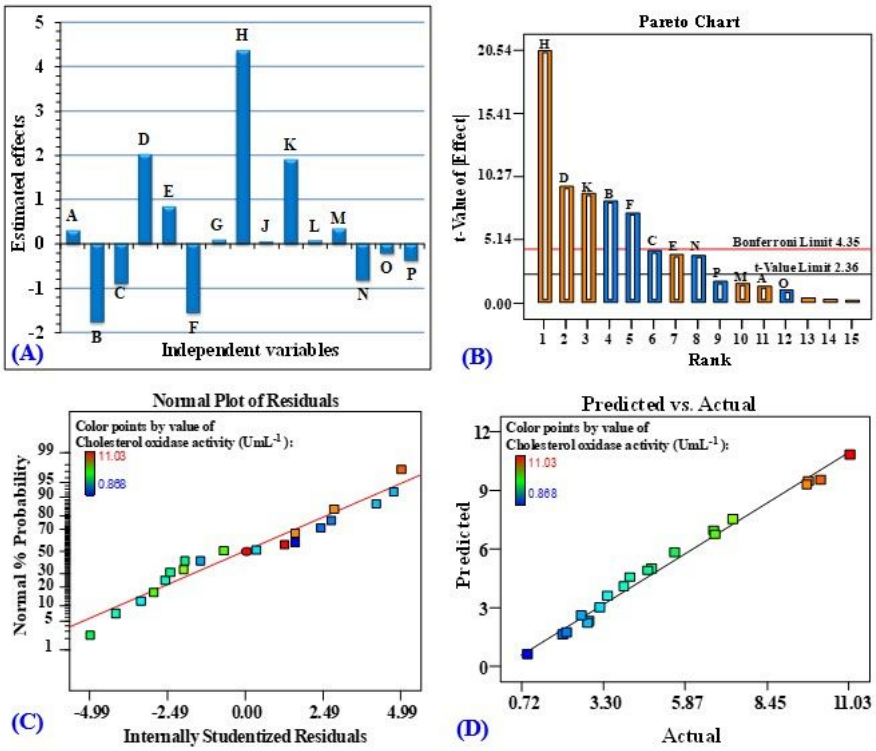

Figure 6

A) The main effects of the variables, B) The Pareto chart shows the order of significance of each variable, C) The normal probability plot of the residuals, D) Correlation between the experimented and predicted values for cholesterol oxidase production by Streptomyces anulatus strain NEAE-94 determined by the first-order polynomial equation.

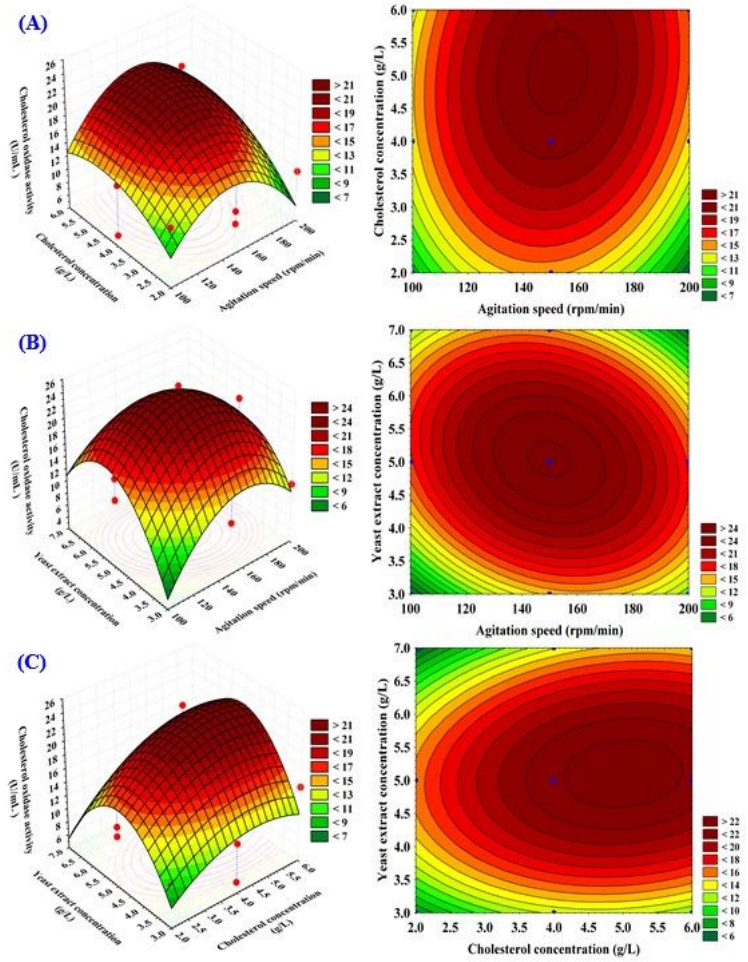

Figure 7

A-C. Three-dimensional response surface plots showing the effect of agitation speed, cholesterol, yeast extract concentration and their mutual effects on the cholesterol oxidase production.