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ORIGINAL ARTICLE

Isolation, molecular identification and statistical optimization of culture condition for a new extracellular cholesterol oxidase-producing strain using response surface methodology

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Abstract The current work details the screening of about 100 isolates from various soil samples, from which 1 isolate was finally selected based on the productivity of cholesterol oxidase. Further biochemical identification tests and 16S rRNA gene sequencing identified this isolate as *Streptomyces badius*. A preliminary culture media optimization was carried out using the initial screening method of Plackett-Burman. Then, a Box-Behnken design was employed to investigate the optimum concentrations of medium components and interactive effects of main variables on cholesterol oxidase production. The regression analysis showed a significant coefficient of determination (R^2) value (91 %), which was in close agreement ensuring a satisfactory adjustment of the proposed model. Maximal enzyme production (2.38 U/mL, i.e., approximately more than 100 % activity in the basal medium) was obtained at: temperature 35 °C; Tween 20 0.1 %; pH 6.5 and yeast extract 0.15 %. This two-stage statistical approach provided rapid identification and integration of key medium parameters for *Streptomyces* sp., resulting in high cholesterol oxidase production.

Keywords Cholesterol oxidase · Isolation · Optimization · *Streptomyces*

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Introduction

Cholesterol and its oxides have been detected in a variety of foods and foodstuffs, especially egg, milk, meat and their processed products (Baticz and Tomoskozi 2002). The first step of cholesterol degradation is performed by cholesterol oxidase (Aparicio and Martín 2008). Bacterial degradation of cholesterol in cholesterol-containing foods may be beneficial for human health to avoid atherosclerosis or coronary heart disease. Cholesterol oxidase-producing microorganisms, including *Arthrobacter* (Chen et al. 2006), *Brevibacterium* (Fujishiro et al. 1990), *Corynebacterium*, *Mycobacterium* (Brzostek et al. 2007), *Nocardia* (Richmond 1973), *Rhodococcus* (Yazdi et al. 2008), *Streptomyces* (Fukuda et al. 1973), and *Streptoverticillium*, have been isolated from a variety of quite different environments. The enzyme has also been found in several Gram-negative bacteria such as *Burkholderia* (Doukyu and Aono 2001), *Chromobacterium* (Doukyu et al. 2008), and *Pseudomonas* (Doukyu and Aono 1998). A cholesterol oxidase from eukaryotic microorganisms such as *Basidiomycetes* and *Schizophyllum* (Fukuyama and Miyake 1979) has also been reported. Cholesterol oxidase (cholesterol: O2 oxidoreductase, EC 1.1.3.6) is a flavin adenine dinucleotide (FAD)-dependent enzyme that is the first enzyme of the cholesterol degradation pathway (Owen et al. 1983). This enzyme is a 3-β-hydroxysteroid oxidase that catalyzes the oxidation of cholesterol to the impermanent intermediate 5-cholesten-3-one with the reduction of molecular oxygen to hydrogen peroxide and the isomerization of a double bond at C5-C6 to C4-C5 of the steroid to produce 4-cholesten-3-one (Smith and Brooks 1976). Cholesterol oxidase plays several important biological roles. Some pathogenic bacteria require cholesterol oxidase to invade their host macrophage, probably because of the ability of cholesterol oxidase to change the physical structure of the membrane by converting cholesterol to cholesten-4-en-3-one (Brzostek et al.

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2007). As these enzymes are unique to bacteria, they constitute a potential target for a new class of antibiotics. Furthermore, cholesterol oxidase exhibits effective insecticidal activity that is very important and critical for pest control strategies in the case of transgenic crops. More recently, it has been demonstrated that Alzheimer's disease β-amyloid selectively oxidizes cholesterol at the C-3 hydroxyl group and catalytically produces 4-cholesten-3-one, therefore it simulates the activity of cholesterol oxidase (Puglielli et al. 2005). The enzyme is also used for the determination of cholesterol concentrations in food, serum and other clinical samples (Naeemi et al. 1995; Richmond 1976).

In this study, we describe the results of our screening program for the isolation and identification of cholesterol oxidase-producing microorganisms from environmental sources. In addition, this is the first report of the two-stage statistical design approach for optimization of cholesterol enzyme production by *Streptomyces badius* as an effective tool for medium engineering. A Plackett-Burman screening design was applied to address the most significant factors that affect enzyme production. Then, a Box-Behnken design was used to determine the optimum level of each of the significant parameters to ensure maximum cholesterol production.

Materials and methods

Isolation of microorganisms

Various soils and water samples from tanneries, dairy, agricultural compost and meat processing factories were collected from several locations in Fars province, Iran. A small amount of each soil and water sample was suspended in sterile 0.8 % NaCl solution, and a portion of the suspension was spread on a screening medium agar plate containing cholesterol as a sole carbon source. This medium contained (g/L): $(NH4)_2SO_4$, 17; K_2HPO_4, 0.25; $MgSO_4{\cdot}H_2O$, 0.25; $FeSO_4$, 0.001; NaCl, 0.005; cholesterol, 2; agar, 18; and 0.5 mL Tween 80. The pH was adjusted to 7.0 before sterilization. The plates were incubated at 30 °C for 3–5 days. Halo-forming colonies were isolated by repeated single-colony isolation on the same medium. Colonies were then picked up and subcultured in the medium plus 0.5 g/L yeast extract. The plates were incubated at 30 °C for 24 h. All the colonies were screened for enzyme production followed by identification of isolated microorganisms using conventional microbiological examinations. The bacterial cultures were centrifuged (7,000*g*) for 10 min. The supernatant obtained was used as the crude enzyme source and was assessed for extracellular activity of cholesterol oxidase. All the bacteria were stored in slant LB agar and, as for *Actinomyces* spp., were maintained on ISP4 medium.

Cholesterol oxidase assay

The assay of cholesterol oxidase activity was based on the conversion of cholesterol to 4-cholestene-3-one (or production of H_2O_2) as described in previous works (Ghasemian et al. 2008, 2009). The rate of H_2O_2 formation was determined by measuring the color developed at 500 nm. The reaction mixture consisted of 3 μmol cholesterol in 1.0 mL 1 % Triton X-100, 300 μmol phosphate buffer (pH 7.0), 0.1 mL enzyme solution, 1.2 μmol 4-aminoantipyrine, 21 μmol phenol, and 20 U horseradish peroxidase in a final volume of 3 mL. The reaction was performed at 37 °C for 10 min and terminated by heating at 100 °C for 3 min. One unit (U) of activity is defined as the amount of enzyme required to produce 1 μmol H_2O_2 under the assay conditions.

Morphological, biochemical and molecular identification of the isolated strain

The morphological, biochemical and physiological traits of the strain were identified according to *Bergey's manual of systematic bacteriology* (Williams et al. 1989). For further identification, 16S ribosomal DNA was analyzed. The 16S rDNA of the strain was amplified using universal 16S ribosomal DNA primers (F: 5′-CAGCCGCGGTAATAC-3′ and R: 5′-ACGGGCGGTGTGTAC-3′). The amplified gene was sequenced and an evolutionary tree calculated from the alignment using the neighbour-joining method, and finally displayed using MEGA software, version 4.1. Bootstrap supports the branching order of ten bacteria. The replication mode of bootstrap was adjusted to 1,000 with 64,231 random seed using the Maximum Composite Likelihood model.

Screening of main factors influences cholesterol oxidase production by new isolate

In order to determine the major factors affecting cholesterol oxidase production by the new isolate, different medium components were evaluated in a two-stage experimental design strategy. In the first stage, a Plackett-Burman factorial design (Plackett and Burman 1946), with seven factors was considered, with each factor being examined at two levels (Table 1): −1 for low level and 1 for high level. These designs gave an output of eight experimental runs (combinations) with seven independent variables. Each component was added in different combinations according to Table 1. A Plackett-Burman experimental design is based on the first order model:

$$Y = \beta_0 + \sum Bixi$$

where Y is the response (enzyme activity), β_0 is the model intercept, β_i is the linear coefficient and *xi* is the level of the

Table 1 Plackett-Burman experimental design for evaluating factors influencing cholesterol oxidase production in the newly isolated strain. Levels of coded variables are in parenthesis

Run	Tween 20 (mL) (X_1)	Yeast extract (%) (X_2)	$(NH4)_2SO_4$ (%) (X_3)	pH (X_4)	Temperature (X_5)	RPM (X_6)	Cholesterol (%) (X_7)	Activity (U/mL)
1	0.1(+1)	0.3(+1)	0.2(+1)	6(−1)	35(+1)	100(−1)	0.1(−1)	0.865
2	0.05(−1)	0.3(+1)	0.2(+1)	8(+1)	25(−1)	150(+1)	0.1(−1)	1.586
3	0.05(−1)	0.15(−1)	0.2(+1)	8(+1)	35(+1)	100(−1)	0.2(+1)	1.038
4	0.1(+1)	0.15(−1)	0.1(−1)	8(+1)	35(+1)	150(+1)	0.1(−1)	0.835
5	0.05(−1)	0.3(+1)	0.1(−1)	6(−1)	35(+1)	150(+1)	0.2(+1)	0.464
6	0.1(+1)	0.15(−1)	0.2(+1)	6(−1)	25(−1)	150(+1)	0.2(+1)	1.426
7	0.1(+1)	0.3(+1)	0.1(−1)	8(+1)	25(−1)	100(−1)	0.2(+1)	1.985
8	0.05(−1)	0.15(−1)	0.1(−1)	6(−1)	25(−1)	100(−1)	0.1(−1)	1.637

independent variable. This model does not describe interaction among factors and is used to screen and evaluate the most important factors that affect the response. In the present work, seven assigned variables were screened in eight experimental designs. All the experiments were performed in triplicate and the average of cholesterol oxidase activity was used as the response (dependent variable).

Optimization of cholesterol oxidase production by new isolate

Response surface methodology (RSM) using a Box-Behnken design experiment was used to increase the mathematical correlation between four independent variables on the production of cholesterol oxidase (Box and Behnken 1960). The variables were studied at low, middle and high concentration levels and were designated as −1, 0 and +1 (coded values), respectively. Table 2 shows the design matrix of 27 trial experiments with four independent variables: temperature, Tween 20, pH and yeast extract. For the four factors, the equation is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{14} X_1 X_4 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2$$

where Y is the predicted response, β_0 is model constant; X_1, X_2, X_3 and X_4 are independent variables; β_1, β_2, β_3 and β_4 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are cross product coefficients and β_{11}, β_{22}, β_{33} and β_{44} are the quadratic coefficients. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Minitab 15 software (version 15, Minitab, State College, PA) was used for the analysis of the experimental data obtained. Experiments were performed in triplicate, and average cholesterol activity was used as the response.

Results

Isolation of microorganism

In the first step, 98 soil samples were collected from different sources. More than 140 colonies were isolated in screening medium containing cholesterol as the sole carbon source. The selected strains were examined for cholesterol oxidase production. The culture broths were assayed for extracellular activity of cholesterol oxidase and one of them, strain 44B (isolate from meat processing factories) showed the highest ability for production of cholesterol oxidase. Maximum level of enzyme production in supernatant of 44B culture was reached to 1.4 U/mL in 3 days. Therefore, strain 44B was chosen as cholesterol oxidase-producer and was considered for further studies.

Identification of isolated strain

The initial identification of microbiological tests revealed that the isolated strain was a rod-shaped mesophilic Gram-positive bacterium. The result of some biochemical tests are listed in Table 3. This strain was also confirmed by phylogenetic analysis. The 16S rDNA of the strain was amplified using universal 16S ribosomal DNA primers. The amplified fragment was sequenced and deposited in GenBank under accession number JF800189.1. The phylogenetic tree based on the 16S rDNA sequence of 800 bp drawn using the neighbor- joining method, showed that the strain is located within the class actinobacteria. This phylogenetic tree is illustrated in Fig. 1. The highest level of sequence homology was shown by *Streptomyces badius* (FJ486408.1) with a similarity of 98 %.

Evaluation factors affecting enzyme production using a Plackett-Burman design

Following the one-factor-at-a-time method, the effect of basal media type, nitrogen source (tryptone, yeast extract

Table 2 Box-Behken experimental design representing the response of cholesterol oxidase enzyme activity as affected by temperature, Tween 20, pH and yeast extract. R^2=91.19 %, R^2(adj)=80.92 %

Run	Temperature (X_1)	Tween 20 (mL) (X_2)	pH (X_3)	Yeast extract (%) (X_4)	Activity (U/mL)	
					Measured	Predicted
1	30(0)	0.1 (0)	5 (−1)	0.15 (−1)	1.4684	1.48422
2	35(+1)	0.1 (0)	5 (−1)	0.3 (0)	1.1436	1.30064
3	30(0)	0.1 (0)	6.5 (0)	0.3 (0)	1.8497	1.79496
4	30(0)	0.1 (0)	6.5 (0)	0.3 (0)	1.7668	1.79496
5	30(0)	0.15 (+1)	5 (−1)	0.3 (0)	1.2652	1.17411
6	35(+1)	0.1 (0)	6.5 (0)	0.45 (+1)	1.9817	1.90362
7	30(0)	0.1 (0)	8 (+1)	0.45 (+1)	1.6356	1.51869
8	30(0)	0.05 (−1)	6.5 (0)	0.45 (+1)	2.0518	2.16176
9	25(−1)	0.1 (0)	6.5 (0)	0.15 (−1)	1.3640	1.48574
10	25(−1)	0.1 (0)	5 (−1)	0.3 (0)	1.8041	1.76490
11	30(0)	0.15 (+1)	6.5 (0)	0.45 (+1)	1.5338	1.55631
12	30(0)	0.1 (0)	5 (−1)	0.45 (+1)	1.8387	1.84962
13	30(0)	0.1 (0)	8 (+1)	0.15 (−1)	1.5492	1.43719
14	30(0)	0.05 (−1)	6.5 (0)	0.15 (−1)	1.4854	1.52050
15	25(−1)	0.05 (−1)	6.5 (0)	0.3 (0)	1.5951	1.51515
16	25(−1)	0.15 (+1)	6.5 (0)	0.3 (0)	1.4714	1.51674
17	30(0)	0.15 (+1)	6.5 (0)	0.15 (−1)	1.8031	1.75067
18	25(−1)	0.1 (0)	8 (+1)	0.3 (0)	1.0167	0.91723
19	35(+1)	0.1 (0)	6.5 (0)	0.15 (−1)	1.7041	1.69606
20	30(0)	0.05 (−1)	8 (+1)	0.3 (0)	1.0380	1.17277
21	35(+1)	0.15 (+1)	6.5 (0)	0.3 (0)	1.5431	1.52195
22	30(0)	0.15 (+1)	8 (+1)	0.3 (0)	1.5072	1.60419
23	35(+1)	0.05 (−1)	6.5 (0)	0.3 (0)	2.0452	1.89881
24	30(0)	0.05 (−1)	5 (−1)	0.3 (0)	2.0342	1.98081
25	25(−1)	0.1 (0)	6.5 (0)	0.45 (+1)	1.6733	1.72508
26	30(0)	0.1 (0)	6.5 (0)	0.3 (0)	1.7684	1.79496
27	35(+1)	0.1 (0)	8 (+1)	0.3 (0)	1.6736	1.77036

and casein), and surfactant (Tween 80, Tween 20 and Triton X-100) on enzyme production was studied. Maximum production was obtained using a Tween 20 and yeast extract combination. The type of nitrogen source and surfactant used for culturing this bacterium played an important role in enzyme production. In medium lacking cholesterol, cholesterol oxidase production was low; however, enzyme production increased after addition of cholesterol (data not shown). Consequently, seven factors were selected to optimize previous media in subsequent studies.

A sequential optimization strategy based on a statistical experimental design was employed to enhance the production of cholesterol oxidase by the local isolated strain. The first step deals with screening for culture parameters as well as nutritional factors affecting growth of the isolated strain with respect to cholesterol oxidase production. The second step is to optimize the factors that control the enzyme production process.

In the first step, the cholesterol-oxidase-producing medium was optimized by the Plackett-Burman design.

Table 3 Biochemical properties of the isolated strain

Property	Result
Catalase	−
Gram staining	+
Starch hydrolyses	−
DNase	−
Maltose	+
Glucose	+
Glycerol	+
Manitol	+
Galactose	+
Nitrate reduction	−
Urea hydrolysis	−
Casein	+
Motility test	−
Indol production	−
Voges-Proskauer	−
Methyl red	−

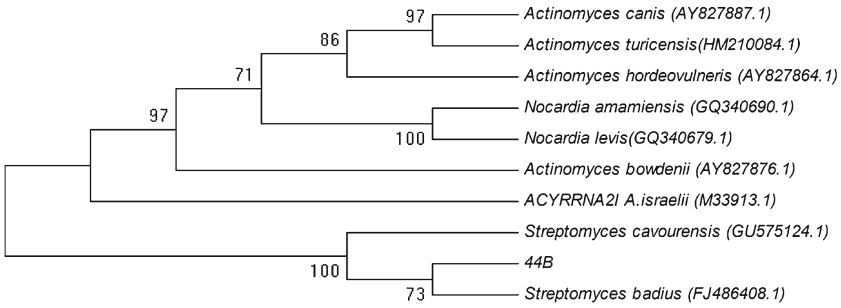

Fig. 1 Phylogenetic tree showing the position of strain 44B based on 16S rDNA sequence and related organisms. The tree was constructed using the neighbor-joining algorithm. Bootstrap values (>50 %) based on 1,000 replications are shown at the nodes of the tree

Seven different factors that were important for enzyme production were chosen for this optimization process. As shown in Table 1, the average value of cholesterol oxidase activity in different trials was determined as 1.23 U/mL. The main effect of each variable on cholesterol oxidase activity was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (−1) of that factor. Results of different trials indicate a wide variation from 0.46 to 1.98 U/mL cholesterol activity. This variation reflects the importance of medium optimization to attain higher productivity. The analysis of the data from Plackett-Burman experiments involved a first order (main effects) model. The main effects of the examined factors on enzyme activity were calculated and are presented graphically in Table 4. The main effect on enzyme production of the factors examined was that all variables had a positive effect on cholesterol oxidase activity except cholesterol and $(NH_4)_2SO_4$, which contributed negatively. A polynomial model describing the correlation between the seven factors and cholesterol oxidase activity can be presented as follows:

$$Y = 1.575 + 0.079X_1 + 0.045X_2 - 0.028X_3 + 0.18X_4 + 0.065X_5 + 0.01X_6 - 0.076X_7$$

According to calculated main effects and *F-test* (Table 4), temperature, Tween 20, pH and yeast extract were found to be the most significant variables influencing cholesterol oxidase activity. Cholesterol was used as the inducer. In preliminary experiments, the concentration of cholesterol was 0.2 %. A cholesterol concentration higher than 0.2 % did not increase enzyme productivity; possibly, the high concentration of cholesterol would reduce the production of cholesterol oxidase. The significant variable cholesterol has a negative effect on cholesterol oxidase production; although it will be kept at 0.2 % value in order to bringing maximum cholesterol oxidase activity. The insignificance effect of $(NH_4)_2SO_4$ may be due to the inorganic nature of ammonium ions, whereas organic nitrogen source (yeast extract) have some carbohydrate and fatty acids that enhance cholesterol oxidase production (Kreit et al. 1994).

Table 4 Statistical analysis of Plackett-Burman design showing Effect, MS and F for each variable on cholesterol oxidase production

Variable	Effect	MS	F
Tween 20 (X_1)	0.079	0.0125	28.66
Yeast extract (X_2)	0.045	0.004	9.12
$(NH_4)_2SO_4$ (X_3)	−0.028	0.00162	3.77
Temperature (X_4)	0.18	0.0651	150.57
pH (X_5)	0.065	0.008	18.34
RPM (X_6)	0.01	0.00064	0.0006
Cholesterol (X_7)	−0.076	0.0115	2.64

Optimization of cholesterol oxidase production by Box-Behnken

The data from the Box-Behnken experiments are analyzed in Table 5. The polynomial model describing the correlation

Table 5 Statistical analysis of Box-Behnken design

Variable	Coefficient	SE coefficient	T	*P*-value
Constant	1.79497	0.07184	24.985	0.000
X_1	0.09722	0.03592	2.706	0.019
X_2	−0.09382	0.03592	−2.612	0.023
X_3	−0.09449	0.03592	−2.630	0.022
X_4	0.11172	0.03592	3.110	0.009
X_1^2	−0.11324	0.05388	−2.102	0.057
X_2^2	−0.06856	0.05388	−1.272	0.227
X_3^2	−0.24344	0.05388	−4.518	0.001
X_4^2	0.02090	0.05388	0.388	0.705
X_1X_2	−0.09461	0.06222	−1.521	0.154
X_1X_3	0.32935	0.06222	5.294	0.000
X_1X_4	−0.00795	0.06222	−0.128	0.901
X_2X_3	0.30953	0.06222	4.975	0.000
X_2X_4	−0.20891	0.06222	−3.358	0.006
X_3X_4	−0.07098	0.06222	−1.141	0.276

between four factors and cholesterol oxidase activity could be presented as follows:

$$Y = 1.79497 + 0.09722X_1 - 0.09382X_2 - 0.09449X_3 + 0.11172X_4 + 0.32935X_1X_3 + 0.30953X_2X_3 - 0.20891X_2X_4 - 0.24344X_3^2 + 0.02090X_4^2$$

where X_1, X_2, X_3 and X_4 are temperature, Tween 20, pH and yeast extract, respectively.

As shown in Table 2, cholesterol oxidase yield of experiments no. 8 and 24 were 2.16 and 1.98 U/mL, respectively. The regression analysis of the data revealed a coefficient of determination (R^2) value of 0.9119, with an adjusted R^2 value of 0.8092, which are in close agreement ensuring a satisfactory adjustment of the proposed model with more than 91 % variability in response being explained by the proposed model and only 8.9 % of the total variance not being explained by the model (Table 2). The adjusted determination coefficient (Adj R^2=0.8092) was also satisfactory to confirm the significance of the model. This indicated that the equation used was a suitable model with which to describe the response of the experiment pertaining to cholesterol oxidase production. To determine the optimal condition of cholesterol oxidase production and the relationship between the response and the significant variables, statistical analyses of variance (ANOVA) was performed through a joint test of four parameters (Table 6). ANOVA for cholesterol oxidase production shows that the fitted second-order response surface model is highly significant with *F*-test= 6.46 (*P*=0.005) as shown in Table 3.

The *P*-value is used for the evaluation of model significance. A very significant model has a *P*-value below 0.01 and a significant model below 0.05.

At the model level, the correlation trial for the evaluation of the regression equation is the multiple correlation coefficient *R* and the determination coefficient R^2. The closer *R* is to 1, the greater the correlation between the calculated and the predicted values (Abdel-Fattah et al. 2005). In this analysis, the value of *R* was 90.97 % for activity of cholesterol oxidase, which shows a good correlation between calculated and predicted values. The value of the determination coefficient is R^2=0.91 for cholesterol oxidase activity.

The optimal levels of the four factors were: temperature, 35 °C; Tween 20, 0.1 %; pH, 6.5 and yeast extract, 0.15 %. Surface plots were obtained and analyzed when data from the experimental design were supplied into the Minitab design software. In these results, the surface plot represents the effect of two independent variables on enzyme production (Fig. 2). The results of ANOVA showed that the linear term of the polynomial model was highly significant. The value of the lack-of-fit *F-test* and lack of fit *P*-value were found to be 0.08 and 0.998 respectively, which implies that the lack-of-fit is non-significant. Non-significant lack-of-fit means that the model fits (Table 6).

Results of regression analysis indicated a significant difference between the four variables in cholesterol oxidase production. However, analysis of four surface plots indicated that the interaction of the two variables temperature and pH was very high. Meanwhile, interaction of pH and yeast extract was low. There were good and positive correlations between temperature and enzyme production. Any increase in temperature led to more enzyme production. On the other hand, an increase of yeast extract led to a decrease in enzyme production (Fig. 2).

Discussion

Cholesterol oxidase enzyme was first isolated from *Nocardia erythropolis* and its role in cholesterol oxidation was confirmed (Richmond 1973). Cholesterol oxidases are also produced by several microorganisms including some *Streptomyces* species, i.e., *S. lavendulae*, *S. polychromogenes*, *S. parvus*, *S. virginiae*, *S. violascens* which are found in various environments (Chauhan et al. 2009; Praveen et al. 2011; Li et al. 2010; Kim et al. 2002; MacLachlan et al. 2000). In this study, a total of 144 colonies that were able to grow in cholesterol medium were isolated. Strain 44B had the highest ability to produce cholesterol oxidase and was

Table 6 ANOVA for the model that represents cholesterol oxidase activity from *Streptomyces*

Source	DF	Seq SS	Adj SS	Adj MS	F	*P*-value
Regression	14	1.92366	1.92366	0.137404	8.87	0.000
Linear	4	0.47597	0.47597	0.118991	7.69	0.003
Square	4	0.39980	0.39980	0.099949	6.46	0.005
Interaction	6	1.04789	1.04789	0.174649	11.28	0.000
Residual error	12	0.18580	0.18580	0.015483		
Lack-of-fit	10	0.18131	0.18131	0.018131	8.07	0.115
Pure-error	2	0.00449	0.00449	0.002247		
Total	26	2.10946				

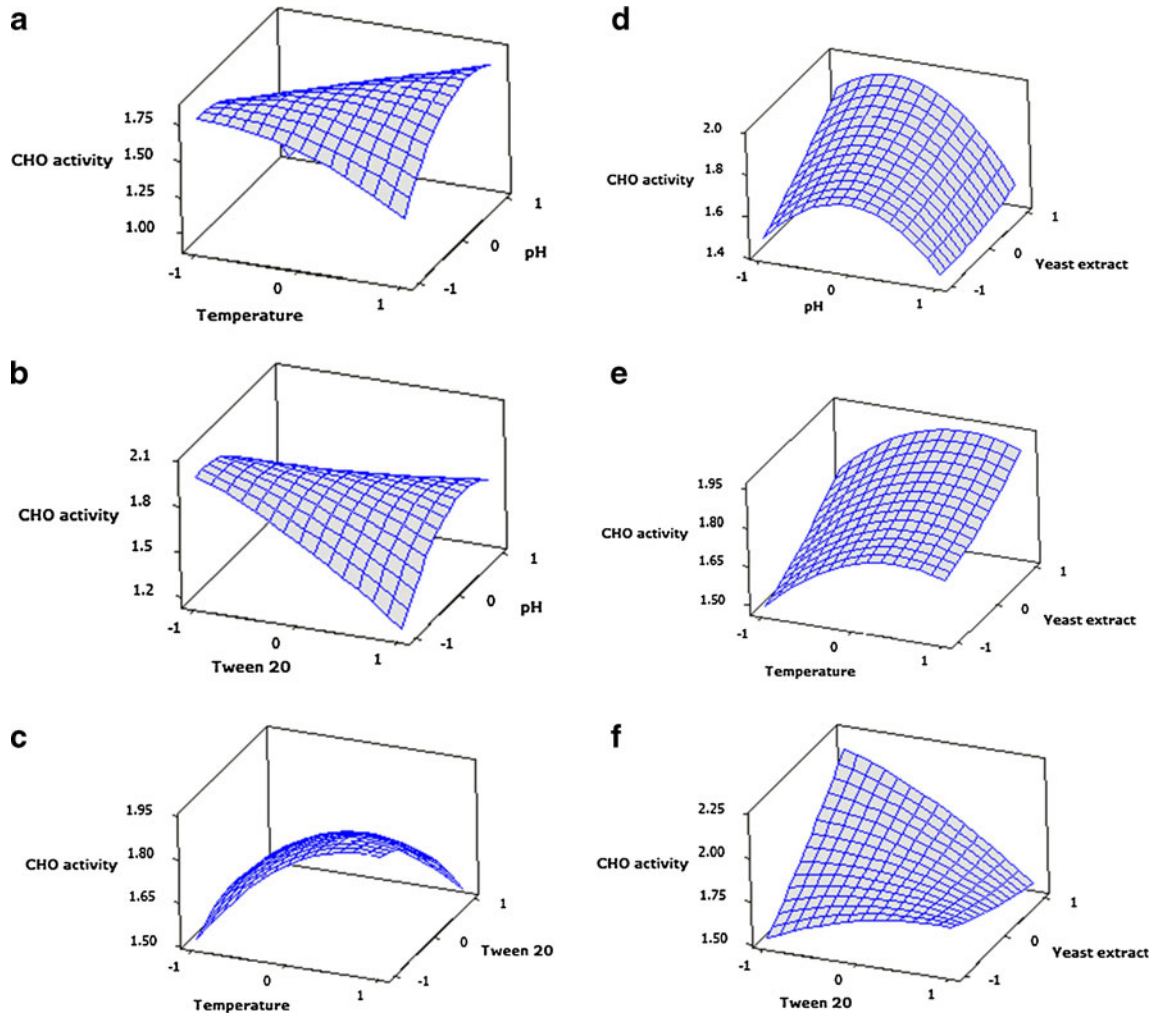

Fig. 2 Response surface plots, main effects plots, and interaction plots showing the effects on cholesterol oxidase enzyme activity (U/mL) by isolated strain of either culture medium components of yeast extract and Tween 20 or environmental effects including temperature and pH. **a** Effect of yeast extract and pH; **b** effect of Tween 20 and temperature; **c** effect of yeast extract and Tween 20; **d** effect of yeast extract and temperature; **e** effect of pH and Tween 20; **f** effect of pH and temperature. In each plot, the other values were held at zero level

selected for further studies. Biochemical and molecular analyses showed that strain 44B belongs to the genus *Streptomyces badius*. As far as we know, no research has so far been conducted concerning the isolation of cholesterol oxidase from *Streptomyces badius*. Cholesterol oxidases from *Streptomyces* species have been reported to have more desirable properties than those from other microorganisms due to lower cost of production, better stability and longer shelf life (Lolekha and Jantaveesirirat 1992). Moreover, *Streptomyces* and *Brevibacterium* have been recognized as major sources of cholesterol oxidase for serum cholesterol determination by the kinetic method.

Although cholesterol oxidases have been described as cell-bound enzymes, some bacteria such as *Rhodococcus* spp. produce both cell-bound and extracellular enzyme. Cell-bound cholesterol oxidases have an amphipatic structure, consisting of an active and an anchor domain. Therefore, it is necessary to use a detergent such as Triton-X 100 or Tween 80 to release the cell-bound enzyme. However, *Streptomyces* commonly secrete cholesterol oxidase into the culture medium as an extracellular protein and there is no need to use detergent, which may affect its structure and activity, to release the enzyme.

Plackett-Burman design is a well-established and widely used statistical design method for the screening of the nutritional components of media in shake flask cultures (Ghasemi et al. 2012). The variables screened by Plackett-Burman design were further optimized using a three-level four-factorial Box-Behnken design methodology. Our studies using the Plackett-Burman design showed that the concentrations of yeast extract and temperature in the medium were important for production of cholesterol oxidase. Application of an appropriate nitrogen source was very important for optimal production of cholesterol oxidase. Among organic and inorganic nitrogen sources, it was revealed that an organic nitrogen source (yeast extract) had

more influence on cholesterol oxidase activity than the inorganic nitrogen source $(NH_4)_2SO_4$. In general, cholesterol oxidase production was enhanced far more by using organic nitrogen than inorganic nitrogen. This may be because organic nitrogen contains most kinds of amino acids and growth factors for the growth of bacterium that could be metabolized directly by cells, consequently promoting cholesterol oxidase production (Chauhan et al. 2009). According to Lee et al. (1997), research on *Rhodococcus equi* no. 23 showed that yeast extract at 0.4–0.5 %w/v was the best nitrogen sourcefor producing cholesterol oxidase. In a similar study, *Rhodococcus equi* 2C supported maximum production of enzyme with yeast extract at 0.3 %w/v (Yazdi et al., 2001). In some research, ammonium salts have shown the highest effect on cholesterol oxidase production by *Arthrobacter simplex* (Liu et al. 1988).

Our first screening procedure showed that cholesterol can be used as a sole carbon and energy source. This finding is inconsistent with that of Chauhan et al. (2009), who reported that *S. lavendulae* is a constitutive producer of cholesterol oxidase, which means cholesterol cannot support the growth of microorganism. On the other hand, some researchers have reported that cholesterol can be used as both energy and carbon source. Lee et al. (1997) assessed different carbon sources on *Rhodococcus equi* and discovered cholesterol to be an inducer of cholesterol oxidase at 0.1 %w/v. In another study, they also evaluated various concentrations of cholesterol (0.52–1.08 % w/v), on *R. equi* no. 23, and deduced that highest productivity of cholesterol oxidase (0.24 U/mL), was achieved at 0.918 %w/v cholesterol (Lee et al. 1999). However, Yazdi et al. (2001) found cholesterol as a best inducer at 0.15 %w/v for production of enzyme by *R. equi* 2C. Moreover, Kreit et al. (1994) assessed some fatty acids on *Rhodococcus* sp. GK1 and found that hexanoate as sole carbon source yielded the highest cholesterol oxidase production at 100 U/g dry weight.

As can be seen in Fig. 2a,c,e, maximum cholesterol oxidase was obtained at incubation at 30 °C, while incubation at lower and higher than 30 °C gave rise to a lower content of cholesterol oxidase. These findings are in agreement with almost all authors, who report the best temperature for cholesterol oxidase production as about 30 °C in *Rhodococcus* sp., *Brevibacterium* sp. and *Bordetella* sp. (Lashkarian et al. 2010; Lin et al. 2010; Salva et al. 1999). However, the time of incubation needed to obtain high yields of cholesterol oxidase as reported by other researchers varied. In our study, maximum cholesterol oxidase production was achieved after 48 h of incubation, and then declined with more incubation time. Yazdi et al. (2001) and Chauhan et al. (2009) reported that maximum cholesterol oxidase production was achieved after 96 and 72 h of incubation for *S. fradiae* and *S. lavendulae*, while Wang et al. (2008) and Ahmad and Goswami (2012) reported 96 and 72 h of incubation for two different strains of *Rhodococcus*.

Figure 2a, b shows the effects of various initial pH values on cholesterol oxidase production. An initial pH of 6.5 supported the highest production of cholesterol oxidase (2.05 U/mL). In contrast, Yazdi et al. (2001) studied the effects of various initial pH on *Rhodococcus* sp., and found that pH 8 was optimum, whereas Lee et al. (1997) reported an initial pH of 7.0 as optimum for enzyme production from *Rhodococcus* sp. 2C. In another study, Chauhan et al. (2009) reported an initial pH for cholesterol oxidase production in *S. lavendulae* of 7.5, which differs from our findings.

Our results show that supplementation of the medium with Tween 20 had a positive effect on cholesterol oxidase production. In contrast, Doukyu et al. (2008) reported that cholesterol oxidase production of *Nocardia erythropolis* and the other *Pseudomonas* sp. were not so high in the presence of detergents, whereas Yazdi et al. (2001) reported that Tween 20 promotes cholesterol oxidase production. The proposed mechanism for enhancement of the addition of such surfectants (e.g., Tween 20) is promotion of both uptake and exit of compounds from the cell through modification of plasma membrane permeability. Furthermore, Tween 20 contributes to disperse cholesterol, which is very water insoluble (Asther et al. 1987).

According to the results presented here, the conditions and the optimized medium for maximum cholesterol oxidase activity of 2.05 U/mL were 30 °C; Tween 20, 0.05 %; pH, 6.5 and yeast extract, 0.45 %. In comparison to other researchers, our experiment showed good cholesterol oxidase production as reported by Wang et al. (2008) and Chauhan et al. (2009) of 1.5 U/mL and 1.4 U/mL for maximum cholesterol oxidase activity in *Rhodococcus* sp and *S. lavendulae*, respectively, while Yazdi et al. (2001) reported 3.14 U/mL for *S. fradiae*.

In conclusion, production of a relatively high quantity of extracellular cholesterol oxidase in a simple medium and in a relatively short time without using any detergent for enzyme extraction are the chief advantages of cholesterol oxidase production by *Streptomyces badius*. The level of production of cholesterol oxidase can be improved by changing fermentation conditions. These properties make *Streptomyces badius* a good candidate for industrial production of cholesterol oxidase, and further characterization of the biochemical and structural properties of the enzyme are warranted.

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