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# Molecular Identification of Cholesterol Oxidase Enzyme-Producing *Streptomyces* Bacteria in Soil of Lorestan Province, Iran

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**ABSTRACT**

*Bacterial Cholesterol oxidase (CHO) is a monomeric, bi-functional and Flavin adenine dinucleotide-linked enzyme that catalyzes oxidation and isomerization cholesterol in the presence of molecular oxygen. Recently, powerful pesticide properties of these enzymes have been discovered on pest and in agriculture was used as a new generation of biological pesticides in cotton plants. This study aimed to isolate and molecular identification of Streptomyces bacteria producing CHO from Lorestan soil to provide biological pesticide in future. A total of 100 samples were collected from garden and agricultural land soils of Lorestan province. First, samples were cultured in cholesterol-containing medium as an alone carbon source. Identification of grown bacteria was performed using the morphological characteristics, biochemical and microbial tests. Then, the colorimetric and browning of culture medium confirmed the activity of CHO. Then, the activity of cholesterol oxides was assayed under various temperature and pH. Obtained strains were confirmed by amplification of 16s rRNA gene using PCR technique and sequencing. A total of three producing cholesterol oxidase bacterial samples were isolated. The results showed that obtained strains belong to Streptomyces. The activity of cholesterol oxidase enzyme was confirmed by the colorimetric, and browning of culture medium tests. Finally, the molecular identity of the bacterial species was detected by sequencing PCR products. The results showed that the bacterial species, Streptomyces, isolated from the soil is capable of producing cholesterol oxidase enzyme.*

**Key words**: Molecular identification, Cholesterol oxidase enzyme, *Streptomyces*, Soil

## INTRODUCTION

Cholesterol oxidase is a two-functional microbial enzyme containing Flavin adenine dinucleotide that belongs to the oxidoreductase family [1]. The cholesterol oxidase enzyme catalyzes the oxidation isomerization reaction of cholesterol to 4-cholesten-3-one associated with revival of oxygen to hydrogen peroxide. This reaction is the first stage in decomposition of cholesterol and cholesterol-derived compounds [2]. The substrate and product of this enzyme have essential biological activities in the body. Cholesterol is one of the most important and major steroid compounds in metabolism of the animals, which is not only considered as an important part of the lipoproteins of the cell plasma membrane, but also a precursor for synthesis of many of the major biological steroids such as biliary acids and various steroid hormones [3].

The cholesterol oxidase is a commercial enzyme with extensive applications in medicine, agriculture, food industry, and pharmacy [4]. One of the most important applications of the cholesterol oxidase enzyme in agriculture is to

produce the fourth generation of the biological pesticides for the pest control of the agricultural crops such as corn, tobacco, and cotton, which can be applied for producing these transgenic plants [5, 6].

Introduction of the fourth generation of pesticides was begun as soon as designing and producing the pest-resistant plants using gene transfer was proposed. At first, the most important produced transgenic plants were based on the *Bacillus thuringiensis* bacterium's gene transfer; however, since a large number of the insects could resist against this bacterium, the scientists also made scientific investments on other compounds. Avidin, ascorbate oxidase, and cholesterol oxidase are some of the fourth generation biological pesticides. The BT toxin's action mechanism is through connecting to the specific receptor in the midintestinal epithelium membrane of the insects; although, some insects might not have such specific receptor in the midintestine, leading to their resistance to this toxin. The cholesterol oxidase lethal effect mechanism is related to the cholesterol oxidation in the midintestinal epithelium membrane of the insects, leading to the physical and structural demolition of the membrane and, as a result, the membrane dysfunction causes the insects' death [6]. Due to the different action mechanisms, the cholesterol oxidase was introduced as a new generation of the biological pesticides, compared to BT [7].

The insecticide property of this enzyme was identified for the first time in early 1993 by researchers of the Department of Agriculture of Monsanto Company in the United States. The cholesterol oxidase enzyme is a protein with strong insecticide ability against the larvae of pests of the agricultural products. The cholesterol oxidase with a strong pesticidal activity (50% lethality in less than 21 ppm) on the boll weevil larva is the most important economic pest of common. Studies on lepidopteran have shown that this enzyme has appropriate pesticidal activity against *Helicoverpazea*, *Pectinphoragossypiella*, and *Heliothisarmigera* [5, 6]. The lethal and various effects of cholesterol oxidase on the pests are caused by reducing the pest's population through the adult female insects' infertility and death and also through the lethal effects on larvae of the pests (especially boll weevil and cotton boll glass); thus, this enzyme is widely used to control the agricultural pests, especially in producing the transgenic products [6, 7].

Considering the protein structure of the cholesterol oxidase enzyme, it is denatured and deactivated, like many other proteins, through heating and is digested in the gastrointestinal tract of the humans and animals by gastric juice; therefore, cloning its gene in cotton and other agricultural products is not harmful for human and animal. [8].

The transgenic plants with cholesterol oxidase gene are not only considered as a suitable instrument for controlling the target insects and pests, but also have numerous advantages for social, environmental, and economical fields as well as human health through reducing the need for the use of chemical insecticides.

Compared to the enzymes derived from the plant and animal resources, the microbial enzymes have many advantages including diversity of catabolic activities, lower production costs, higher production, abundant and continuous resources, and more durability [9].

The cholesterol oxidase enzyme is produced in the gram-positive and gram-negative bacteria in three extracellular, intracellular, and membrane-bound forms [1]. The cholesterol oxidase-producing bacteria are mainly divided in two categories: 1) non-pathogenic bacteria, and 2) pathogenic bacteria. The non-pathogenic bacteria use cholesterol as a source of carbon for growth, while the pathogenic bacteria need the cholesterol oxidase to infect the host [10].This enzyme was isolated, for the first time in 1944, from the *Nocardia erythropolis* bacterium and is currently produced from a wide range of microorganisms in the environment such as *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Schizopyllum*, *Brevibacterium*, *Streptomyces*, *Rhodococcus*, *Burkholderia* etc. [11].

Considering the high commercial value as well as the widespread and increasing applications of the cholesterol oxidase enzyme in various fields of medicine, agriculture, and industry, many researchers have focused in recent years on discovering the ecosystems and natural environments to find new bacteria with high capability to produce the cholesterol oxidase enzyme as the first step for mass industrial production of this enzyme. The present study was aimed to molecularly identify the cholesterol oxidase-producing *Streptomyces* bacterium and comparing the biochemical activities of the cholesterol oxidase enzyme of the native soil bacteria in the province in order to use them as the biological pesticides for cotton.

## MATERIALS AND METHODS

**Sample collection**

A total of 100 soil samples were collected from the farm lands and gardens in the cities of Khorramabad, Poldokhtar, and Borujerd in Lorestan Province, Western Iran. Since the cholesterol oxidase-producing bacteria are aerobic, sampling was performed from the soil's surface to the depth of 5 cm.

**Bacteria isolation**

For better isolation of the bacteria from soil, first, one gram of each soil sample was dissolved in 100 ml of distilled water. In order to prepare the suspension, the samples were placed in the shaker for 30 min; afterwards, 0.1 ml of the supernatant of each sample was removed and inoculated into the mineral agar screening medium, composed of 0.1% cholesterol, 0.25 g $K_2HPO_4$, 0.05 g NaCl, 17 g $NH_4NO_3$, 0.25% $MgSO_4.H_2O$, 0.001 g $FeSO_4.7H_2O$, 0.5 ml Tween 80, 1.8% agar with pH=7 (manufactured by Sigma Co) and then incubated at temperature of 30 °C for 8-12 days. It should be noted that the mineral agar medium contains the cholesterol as the only carbon source, and only the bacteria capable to produce the cholesterol oxidase enzyme can grow in it. At the end of the incubation period, a part of each of the single colonies emerged on the mineral agar medium (containing 0.5% weight/volume of yeast extract) was removed and subcultured in the enriched mineral agar medium and then incubated at temperature of 30 °C for 4 days. This was done in order to purify and prepare the more grown and larger colonies for performing other tests.

**Approving cholesterol oxidase activity of grown colonies**

The two red and brown colony staining methods were used to approve the cholesterol oxidase activity of the grown colonies. In the colony red staining method, first, a solution composed of 0.5% cholesterol, 0.06 g Phenol, 1.7 g 4-Aminoantipyrine, and 3000 units per liter of HRP (horse radish peroxidase) enzyme in 100 mM potassium phosphate buffer with pH=7 (manufactured by Sigma Co) was prepared; then, a filter paper was completely soaked in the solution and placed on the colonies grown on the surface of the plates and, then, incubated at temperature of 30 °C for 24 h. The cholesterol oxidase activity of the tested colonies was approved by producing the red color resulted from formation of the quinoneimine dye. In this method, the bacteria with cholesterol oxidase enzyme oxidize the cholesterol and produce hydrogen peroxide; subsequently, the hydrogen peroxide is combined with phenol and 4-aminoantipyrine, reacts with HRP, and reddens the culture medium by producing the quinoneimine dye [12].

The next stage of the test was brown staining the culture medium using the colonies' strains that reddens the medium and have the cholesterol oxidase activity. In this test, first, the culture medium composed of 1 g cholesterol, 1 g triton X-100, 0.1 g o-dianisidine, and 1000 units per liter peroxidase enzyme in 1 liter of LB agar medium (manufactured by Sigma Co) was prepared. Then, the strains were cultured on the plates and incubated at temperature of 30°C for 24 h. In this test, the cholesterol penetrates into the bacteria, and the bacteria with cholesterol oxidase enzyme oxidize the cholesterol in the culture medium and produce hydrogen peroxide, which reacts with the peroxidase reagent and turns the culture medium's color to brown by forming the azo compound [12].

**Extracting extracellular and intracellular cholesterol oxidase enzymes**

In order to extract the cholesterol oxidase, the enzyme-producing colonies were cultured in 100 ml of LB liquid medium with pH=7 and placed in the shaking incubator at speed of 150 rpm and temperature of 30 °C for 48 h. Then, 20 ml of the culture medium was poured in the sterile falcon and centrifuged at speed of 3500 rpm for 15 min. The supernatant was used as the source of the extracellular enzyme to evaluate the enzyme activity. Afterwards, the solution in the tube was discarded and the cellular plate in the tube's end was washed twice using 0.1 M phosphate buffer with pH=7.2;then, the bacteria's cellular structure was demolished using the ultrasonic device in order to release the intracellular enzyme within the cytoplasm to the outer space. Then, 5 ml of the same 0.1 M phosphate buffer containing 0.1% triton X-100 (manufactured by Sigma Co) was poured in the tube and incubated at temperature of 30 °C for 30 min, and then centrifuged at speed of 3500 rpm for 15 min. The supernatant was used as the source of intracellular enzyme.

**Measuring cholesterol oxidase enzyme's activity**

In order to evaluate the cholesterol oxidase enzyme's activity, the enzyme solution was kept at various pHs and temperatures for 24 h, and then its activity was measured using the modified method of Sasaki et al. [13].In this

method, 0.1 ml of the extracted enzyme solution, 0.4 ml of triton X-100, 1.15 ml of1.0 M phosphate buffer with pH=7.2, 0.3 ml of HRP, 0.1 ml of o-dianisidine, and 0.05 ml of cholesterol (manufactured by Sigma Co) were mixed in a tube. The reaction occurred at temperature of 37 ℃ for 10 min, and then stopped by increasing the temperature up to 100 °C for 3 min. The hydrogen peroxide was produced through oxidation of cholesterol by the cholesterol oxidase enzyme and formed an oxidative coupling with o-dianisidine, and then the increase in absorption was measured at wavelength of 500 nm using a spectrophotometer device. According to the definition, a unit of enzyme activity is equal to the formation of 1 micromol of hydrogen peroxide for one minute at temperature of 37 °C.

**Detection and identification of bacteria**

The primary identification of the bacteria in terms of their genera was performed based on the morphological properties such as shape and colony color and using various microbiological and biochemical tests including gram staining, acid-fast staining, aerobic or anaerobic, motion, catalase, oxidase, urea, lactose, glucose, sucrose (saccharose), xylose, nitrate, citrate, indole, methyl red, growth on the blood agar medium, and MacConkey agar medium.

**Extracting bacteria's genomic DNA**

Determining the species of the aforementioned bacteria was performed using the PCR 16S rRNA molecular method. First, the bacterium was cultured in the LB liquid medium with pH=7, and then incubated in the shaking incubator at speed of 150 rpm at temperature 30°C for 72 h. At the end of this period, 12 ml of the medium was transferred to the sterile falcon and centrifuged at speed of 4500 rpm for 12 min; then, the supernatant was discarded and the bacterial sediment at the falcon's bottom end was used to extract the bacterium's genomic DNA using the DNA extraction kit manufactured by Cinnagen Co. Afterwards, the extracted genomic DNA was transferred to the freezer for storage at temperature of -20 °C.

**16S rRNA gene replication using PCR method**

In order to replicate the 16S rRNA genes using the PCR technique, the following primers were designed and ordered for synthesis to GenFan-Avaran Co: 27-F :( 5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492-R :( 5′-TACGYTACCTTGTTACGACTT-3′).

The PCR reaction was performed with final volume of 25μLcontaining 2.5μL of buffer (10X), 2 mM MgCl2 (50 mM), 3 mM dNTPs (10 mM), 1.5 unit of Taq DNA polymerase enzyme (0.2 unit), 0.4 pmol of primer (10 pmol), 3 μLof DNA sample (manufactured by Cinnagen Co), and 15.45 μL of deionized water.

The PCR reaction was performed under the following temperature conditions: the initial denaturation temperature of 94°C for 4 min followed by 30 cycles, including the denaturation temperature of 94°C for 50 s, junction temperature of 56°C for 60 s, initial replication temperature of 72°C for 60 s, and final replication temperature of 72°C for 7 min in the Eppendorf gradient thermocycler device. Then, the PCR product along with the molecular weight marker underwent electrophoresis on the 1% gel, and subsequently the band was approved using the Biometra gel imaging device. At the final stage, the purified PCR product was transferred to the sterile microtube and sent to the Gen Fan-Avaran Co for sequencing.

## RESULTS AND DISCUSSION

Among 100 soil samples collected across Lorestan province, three strains of the cholesterol oxidase enzyme-producing bacteria were isolated. The initial screening criteria included cholesterol consumption and oxidation using cholesterol oxidase enzyme-producing bacteria as the only source of carbon for growth on the mineral agar, indicating the ability of the bacterium to produce the cholesterol oxidase enzyme.

After sub-culturing the bacteria colonies on the mineral agar medium, it was observed that, in terms of morphology, the grown colonies were those with white color, dry and chalky appearance, and strong soil smell (Fig.1). The gram staining on the cholesterol oxidase-producing bacteria samples showed that the bacillus was gram-positive with mycelium branches (Fig.2).

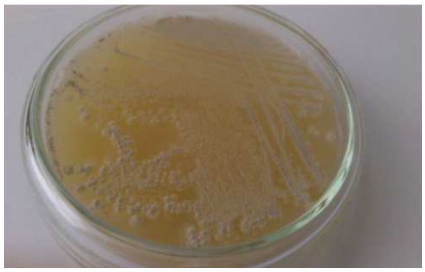

**Figure-1: Cholesterol-oxidizing *Streptomyces* bacterium on enriched mineral agar medium**

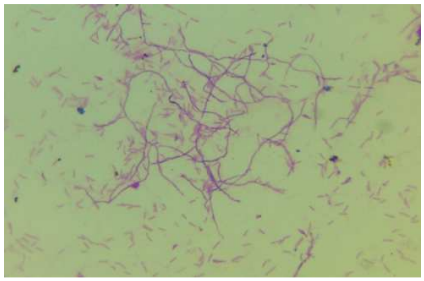

**Figure-2: Gram-positive bacilli smear with mycelium of *Streptomyces* bacteria**

Results of the microbiological and biochemical tests for primary identification of the bacteria's genera showed that the cholesterol oxidase enzyme-producing bacterial samples isolated from the soil belonged to the genus of *Streptomyces* (Table1).

**Table 1. Biochemistry and microbiology test results to identify the primary producing *Streptomyces* cholesterol oxidase**

| Test | Reslut | Test | Reslut | Test | Reslut |
|---|---|---|---|---|---|
| Gram Reaction | + | Urea Hydrolysis | + | Growth in McConkey | - |
| Oxidase | + | Sucrose | + | Haemolysis | - |
| Catalase | + | Lactose | + | | |
| SIM | - | Rhamnose | + | | |
| Acid fast | - | Glucose | + | | |
| Lysozyme Resistance | - | Xylose | + | | |
| MR | - | Casnein | + | | |
| Indol | - | Tyrosine | + | | |
| VP | - | Gelatin | + | | |
| $H_2S$ Prodution | - | Starch | + | | |
| Nitrat Reaction | - | Xanthine | + | | |
| Citrat Uitilization | + | DNase | - | | |

In the next stage, the red and brown staining tests on the medium were positive, confirming the cholesterol oxidase activity of the bacterial colonies (Figs.3 and 4).

**Figure-3: Brown staining of culture medium using cholesterol oxidase enzyme-producing *Streptomyces* bacteria**


**Figure-4: Red staining of culture medium using cholesterol oxidase enzyme-producing *Streptomyces* bacteria**

The isolated *Streptomyces* bacteria have the ability to secrete the enzyme in both extracellular and intracellular forms, yet the extracellular secretion rate is higher. Assessing the effect of various temperatures and pHs on activity and stability of the extracellular cholesterol oxidase enzyme showed that the enzyme's activity occurred in the temperature range of 20-60°C and in pH=5-9, however the highest extracellular enzyme's activity was 3.64 U/ml at temperature of 40°C and pH=7.

**Molecular identification of bacterial species**

The 16s rRNA sequences were replicated using the primer pairs designed with PCR technique. In Figure 5, the results of emergence of the electrophoresis band at region of bp 1500 approves the purity of the PCR products in the genome sample for sequencing. Then, the sequences and genetic analysis were compared using n Blast software available in NBCI website, indicating that the sequences of the bacterial samples isolated from soil had 98% genetic similarity with B5W22-*2Streptomyces* Sp. with access number of EF114310.2, which approves the results of the previous biochemical and microbiological tests based on the bacterial genus of *Streptomyces*.

*Streptomyces* is a genus of the gram-positive, aerobic, motionless, and *Terricolous filamentous* bacteria belonging to the order of the *Actinomycetes* bacteria. The *Streptomyces* colonies have white color, dry and chalky appearance, and strong soil smell [14].

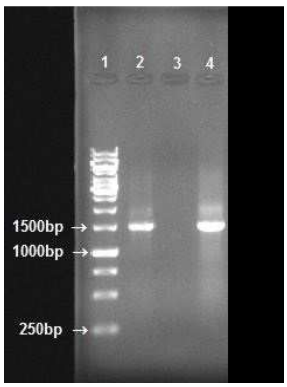


**Figure-5: Gel electrophoresis of PCR 16s rRNA product using primers 27F and 1492R:1. 1kb marker, 2. *Streptomyces* bacteria strains, 3. Negative control, 4. Positive control (*Streptomyces* bacteria strains of gene bank)**

Today, the use of chemical pesticides against the agricultural pests is no longer cost-effective due to the environmental pollutions, effects on human health and products' quality, and pests' resistance. A solution to overcome such problems is to produce transgenic plants resistant against the plant pests, which is considered as one of the most important methods for pest control and currently the developed countries have made massive investments in this regard [15].

The cholesterol oxidase enzyme has strong pesticidal effects on the larva and adult female insect of the pests such as *H. armigera*, boll weevil, and *P. gossypiella*, and due to the presence of cholesterol in membrane of all the eukaryotic cells, there have been no reports so far on the pests' resistance against it [7]. On the other hand, the cholesterol oxidase protein is denatured and deactivated by heating and is digested in the gastrointestinal tract of the humans and animals by the gastric juice; therefore, cloning the gene in cotton and other agricultural products

wouldn't be harmful for human and animals [9] and can be widely used in agriculture for pest control, especially for producing the transgenic products [5].

*Streptomyces* species have the largest genome among the *Actinomycetes* with very high G+C percentage of about 73-69 mol percent. The long genome of *Streptomyces* gives them the ability to produce various enzymes as well sa various secondary metabolites such as antibiotics [16].

In the present study, in order to isolate the cholesterol oxidase enzyme-producing bacteria, the mineral agar screening medium was used with cholesterol as the only source of carbon for growth of the bacteria. After culturing the samples on the initial screening medium, the white and chalky colonies with soil smell grew on the plate's surface, indicating the cholesterol oxidization by the cholesterol oxidase enzyme-producing bacteria. Morphologically, this was a gram-positive bacillus-shaped bacterium with mycelium branches, which was demonstrated by results of the biochemical and microbiological tests to belong to the genus of *Streptomyces*. Then, the test results forred and brown staining of the medium using the isolated *Streptomyces* bacteria confirmed the cholesterol oxidase activity of the colonies as well as the ability of these bacteria to produce the cholesterol oxidase enzyme, which was consistent with previous results [12]. (Investigating the cholesterol oxidase enzyme of the *Streptomyces* isolated from soil in terms of type of secretion (extracellular and intracellular) showed that this bacterium had both extracellular and intracellular secretion, while the extracellular secretion was higher than the other type, which was in agreement with previous studies[17-19].

On the other hand, the cholesterol oxidase enzyme's activity in various pH and temperature conditions was assessed by measuring the optical absorption at 500 nm. The temperature range of the enzyme activity of cholesterol oxidase-producing *Streptomyces* species is commonly between 15-80 °C [16]. Results of the previous studies have also shown that the optimum temperature for activity of the cholesterol oxidase enzyme for the species of *Streptomyces* SA-COO, *S. violaceus*, and *S. fradiae* is 45-80°C, 50°C, and70°C, respectively. Results of the present research showed that the extracellular enzyme activity of the isolated bacterial samples was at the temperature range of 20-60°C, with maximum enzyme activity at 40°C [19-21].

The optimum pH reported for most of the cholesterol oxidase enzyme-producing *Streptomyces* species is between 7 to 8 [17]. Results of the present study showed that the extracellular enzyme's activity was in the range of pH=5 to pH=9 with the maximum activity at pH=7, which were completely consistent with previous [20-22].

Finally, the definitive identification of the cholesterol oxidase enzyme-producing bacteria isolated from soil was accomplished using the PCRrRNA16s technique, and the results of sequencing showed that the isolated cholesterol oxidase enzyme-producing bacteria's strains had 98% genetic similarity with *Streptomyces*B5W22-2.

In the present study, the maximum activity of the extracellular cholesterol oxidase enzyme of the species of *Streptomyces* A, and B was 2.44 U/ml and 2.25 U/ml, respectively, which was higher than the activity of the extracellular cholesterol oxidase enzyme of *S. slavendulae* (NCIM 2499) by 1.140 U/ml. Furthermore, the extracellular enzyme activity in this study was significantly higher that of other cholesterol oxidase enzyme-producing bacteria such as 1.5 U/ml *Arthrobacter* simplex, 0.434COX8-9U/ml *Enterobacter*, 1.28 U/ml *Brevibacterium*, 0.35 U/ml *Pseudomonas*, 0.29 U/ml *Rhodococcus* [23].

Results of the present research showed that the *Streptomyces* bacterium isolated from soil was capable of secreting the cholesterol oxidase enzyme in both intracellular and extracellular forms. According to the aforementioned results, it seems that the *Streptomyces* genus is an appropriate bacterium for industrial and agricultural applications to extract the extracellular cholesterol oxidase enzyme. In the future, the genetic studies for isolating the cholesterol oxidase gene as well as purifying and investigating the optimal conditions for higher production of cholesterol oxidase enzyme would help taking an important step toward the industrial mass production of this enzyme.

Production of the cholesterol oxidase from the native microorganisms bacterial sources in soil is economically cost-effective in comparison with animal sources for some reasons including the ease of collecting the bacteria, easy cultivation, low and simple nutritional requirements for growth of bacteria, rapid growth of bacteria and access to large bacterial mass in short time, more secretion and production of cholesterol oxidase enzyme in bacteria, and easy extraction of cholesterol oxidase enzyme in bacteria. Therefore, identification and isolation of the soil's native cholesterol oxidase enzyme-producing bacteria and microbes with capability of abundant production and secretion

of cholesterol oxidase, determining the optimum conditions for rapid growth of the bacteria for more enzyme production, as well as extraction and purification of the cholesterol oxidase gene for colonizing it in the cotton plant as the biological pesticide to produce the transgenic cotton with the cholesterol oxidase enzyme-encoding gene in order to achieve resistance against the pests essentially require choosing the most appropriate native bacterial strains in terms of expression, secretion, and high activity of this enzyme. Thus, by isolating and identifying the soil's bacterial species, introducing the best strains in terms of mass production of the cholesterol oxidase enzyme, extracting the enzyme and investigating type of its secretion, comparing their biochemical and enzymatic activities, and purifying the cholesterol oxidase enzyme and determining its genetic and molecular characteristics, the present study has taken the first step to produce the transgenic cotton with the cholesterol oxidase enzyme-encoding gene in Iran for biologically fight against the cotton pests in order to solve a part of the cotton farmers' problems and provide healthier products with better quality, higher production rate, and lower cost in the market.

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