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# Cloning, expression, and enzymatic activity evaluation of cholesterol oxidase gene isolated from a native *Rhodococcus sp.*

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## ABSTRACT

*Cholesterol oxidase (CHO) is one of the valuable enzymes that play an important role in: measurement of serum cholesterol, food industry as a biocatalyst and agriculture as a biological larvicide. This enzyme was produced by several bacterial strains. Wild type enzyme produced by Rhodococcus sp. secret two forms of CHO enzyme: extra cellular and membrane bound type which its amount is low and unstable. The goal of the study was cloning, expression, and enzymatic activity evaluation of cholesterol oxidase gene isolated from a native Rhodococcus sp. CHO gene was isolated from native bacteria and cloned into pET23a. In the next step, the construct was expressed in E.coli BL21 and induced by different concentration of IPTG ranges from 0.1 - 0.9 mM. This gene contains 1642 bp and encodes a protein consists of 533 amino acids. It has about 96 % homology with CHO gene isolated from Rhodococcus equi. The high expression was obtained in 0.5 mM concentration of IPTG after 4 hour induction. This recombinant enzyme had a molecular weight of 55 kDa, that secretion of intra cellular type is much more than extra cellular form. The optimum pH and temperature conditions for the recombinant enzyme were 7.5 and 45°C, respectively. CHO enzyme obtained from Rhodococcus sp. is a cheap enzyme with medical and industrial applications that can be produced easily and purified in large scale with simple methods.*

**Keywords:** Cloning, Expression, Enzymatic activity, Cholesterol oxidase gene, *Rhodococcus sp.*

## INTRODUCTION

Cholesterol oxidase (CHO) is a FAD (Flavin-Adenine-dinucleotide) dependent enzyme that catalyzes cholesterol oxidation and produces hydrogen peroxide (H2O2) [1].This enzyme was produced by several bacterial strains such as: *Pseudomonas, Brevibacterium, Streptomyces, Chromo bacterium, Mycobacterium* and *Rhodococcus* [2]. CHO is one of the valuable enzymes that play an important role in: measurement of serum cholesterol and other clinical samples, food industry as a biocatalyst and agriculture as a biological larvicide [3]. This enzyme can tolerate organic solvent and non ionic detergents without decreasing its activity. It exists in two forms: non covalent linked with FAD (class I) and covalently linked with FAD (class II) [3, 4]. Many bacteria require this enzyme for their pathogenicity. They oxidase cellular membrane and has the ability to convert cholesterol to cholest-4-en-3-one and produce H2O2 [5, 6]. As mentioned before, CHO was isolated from several bacteria. More over CHO gene was recently isolated from many bacteria including: *Brevibacterium Sterolicum ATCC21387, Arthrobacter simplex, Streptomyces SA-COO and Rhodococcus sp PTCC1633,* and cloned into appropriate hosts [7].Wild type enzyme produced from genus of *Rhodococcus* such as sp.PTCC1633 and *Rhodococcus sp.* secret two forms of CHO enzyme: extra cellular and membrane bound type which its amount is low and unstable [8]. In the present study, we cloned CHO gene into a suitable host and expressed high recombinant protein .Then enzyme activity in different pH and temperature conditions was measured.

## MATERIALS AND METHODS

***Bacterial strain and plasmid***

*Rhodococcus sp.* was isolated from the Lorestan local soil .*Escherichia coli BL21 plyss* strain and *DH5α* strain were used as a host for cloning and expression of the CHO gene. Plasmid pET23a was used as a vector. Both of bacterial strains as well as plasmid pET23a were purchased from gene bank of Pasteur Institute.

***Enzymes and chemicals***

Restriction endonuclease, *EcoRI, Hind III, pfu* polymerase and *T4 DNA ligase* were all purchased from Fermentas Company and used according to manufacturer's instructions. IPTG purchased from Sigma Company was used for inducing of *Lac* promoter increasing expression of recombinant enzyme. The other reagents were all available in our lab.

***Cloning of cholesterol oxidase gene***

At first, nucleotide sequences of CHO genes available in gene bank were aligned and their homologies with CHO gene isolated from *Rhodococcus* were determined. Then, high similar parts were identified. According to this analysis and on the basis of findings, a pair of primers were designed and used for amplification of CHO gene by PCR method.

Forward primer: 5 – TATAGAATCATGACCGATAGCCGGCGA-3 (***EcoRI*** cleavage site)
Reverse primer 5'- TACCAAGCTTTCACTGGATGTCGGACGAGA-3' (***Hind III*** cleavage site)

PCR assay was performed using above primers and under standard conditions. The reaction min for PCR amplification containing: 1µl DNA template, 2.5IU *pfu* polymerase (Fermentas, Germany), 5 µl 10X *pfu* buffer (containing 50mM Mgcl2), 10 pmol of each primer (1.5µl ), 1.0 µl dNTPs (10mM), and double-distilled water to a final volume of 50 µl. PCR program was as follows: 6 min at 95˚C for initial denaturation, 45 sec at 94˚C, 50 sec at 58˚C, and 60 sec at 72˚C for 30 cycles following 10 min at 72˚C for final extension. PCR product was then analyzed on 0.8% agarose gel and purified using gel extraction kit (Metabion, Germany). Plasmid DNA *(pET23a)* was also extracted using plasmid extraction kit (Roche, Germany). Both of purified PCR product and plasmid DNA was digested by *EcoRI* and *Hind III* restriction endonuclease enzyme for 3h at 37˚C. Thereafter, ligation reaction was carried out using *T4 DNA ligase* at 14˚C over night incubation according to manufactures instruction. This recombinant construct was named "*CHO gene*" and transformed into *E. coli DH5α* competent cells. Resulting transformant was then cultivated on LB agar medium containing 100µg/µl ampicillin at 37˚C over night. A number of growing bacteria was selected and cultured in LB broth. In the next step, extraction of recombinant plasmid was done. In order to check cloned gene it was amplified by PCR using previous primers and sent to Genfanavaran Co. to be sequenced. Obtained nucleotide sequence was analyzed using Blast system from NCBI.

***Expressions of recombinant construct:***

Since *E. coli BL21* showed to be a proper host for gene expression, in this study recombinant plasmid *CHO gene* was extracted from *E .coli DH5α* and then transformed into *BL21plyss* strain competent cells and was plated on LB agar containing 100µg/µl ampicillin and incubated in 37˚C over night. Recombinant bacteria were inoculated on LB broth containing 100µg/µl ampicillin. Five hundred µl of primary over night culture was inoculated in a new LB broth medium consisting of ampicillin. Expression of the recombinant gene was induced when OD of the medium reached to 0.4 – 0.8 at 600nm by adding IPTG up to final concentrations of 0.5mM. After completing 4 hours incubation at 30˚C, 1ml of medium was poured into 1.5ml micro tube and then centrifuged at 12000 rpm for 1 min.

***Molecular characterization of recombinant CHO:***

Recombinant enzyme was analyzed using SDS-PAGE according to Sampson et al method. For this purpose, 1 ml of medium containing bacteria was centrifuged at 12000 rpm for 1 min. Supernatant solution was used as extra-cellular enzyme template. Cellular pellet were also mixed with 100 µl solving buffer and then boiled at 95˚C for 10 min. After centrifugation, 35 µl of supernatant along with 35 µl of plasmid PET23a as negative control and 15 µl of low molecular weight marker were loaded on SDS-PAGE. It must be mentioned that, the supernatant in the second step was applied as a source of intracellular enzyme and cellular pellet was used as a source of inclusion body.

***Measurement of recombinant construct: Enzyme activity***

According to Sasaki method, hydrogen peroxide ($H_2O_2$) generated by cholesterol oxidation of recombinant enzyme was measured [9]. In this reaction, hydrogen peroxide was coupled with 4- amino antipyrine and phenol by peroxidase to generate quinoneimine dye which its absorption was measured in 460 nm. A unit of activity (U) is defined as the amount of enzyme needed to form 1µmol hydrogen peroxide.

***Enzyme stability assessments of recombinant construct:***
Recombinant enzyme stability was evaluated for one hour under different pH and temperature conditions according to method of Sasaki et al.

## RESULTS

Using designed primers for amplifying cholesterol oxidase gene (*CHO gene*), PCR assay was carried out on chromosomal DNA extracted from *Rhodococcus sp.* PCR product was about 1642 bp which was cloned into *pET23a* (Fig. 1).

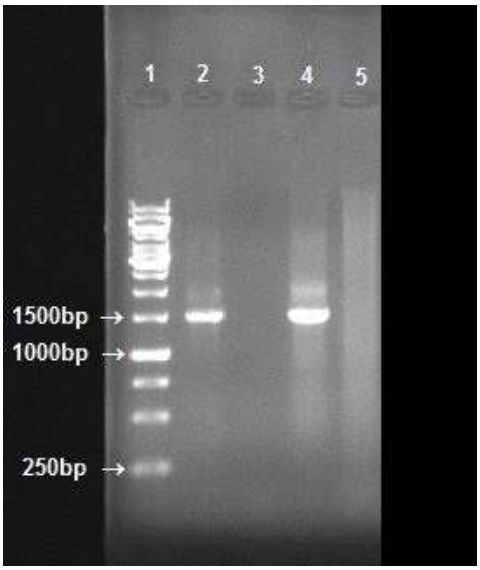


**Fig. 1. Electrophoresis of PCR product resulting from amplification of CHO gene**
*Lane 1:1kb DNA ladder, Lane 2 and 4: CHO gene, Lane 5: Non template control*

Nucleotide sequence of insert fragment was determined using blast system available in NCBI. Analysis of protein coded by *CHO gene* showed that this gene encode a polypeptide consisting of 533 amino acids. The homology and similarity of *CHO gene* with *Rhodococcus equi*, and other *Rhodococcus* was about 98%. Up to 50 constructs were examined for expression. Among these, one of them that had the highest intra cellular CHO activity was selected. SDS-PAGE analysis of *E .coli BL21plyss* harboring pET23a ( CHO) under induction of IPTG indicated that there is a strong band in 55 kDa when was run along with *E. coli BL21+PET23a* as negative control and no band was observed (Fig. 2).

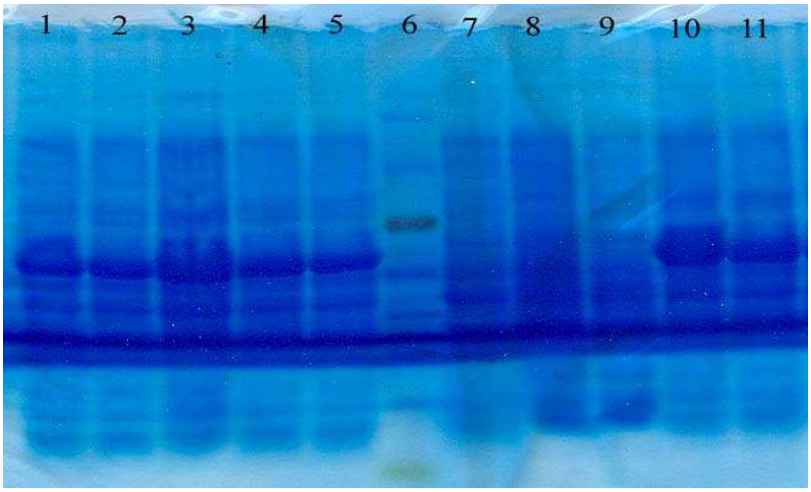


**Fig. 2a. SDS-PAGE gel of recombinant cholesterol oxidase enzyme**

*Lane 1to5: IPTG concentration gradient (0.1, 0.3, 0.5, 0.7, and 0.9), Lane5: Low molecular size marker, Lane7: E.coli Bl21plyss+ pET23a as control negative. Lane9: Extracellular CHO enzyme.Lane10: intracellular CHO enzyme.Lane11: Inclusion body.*

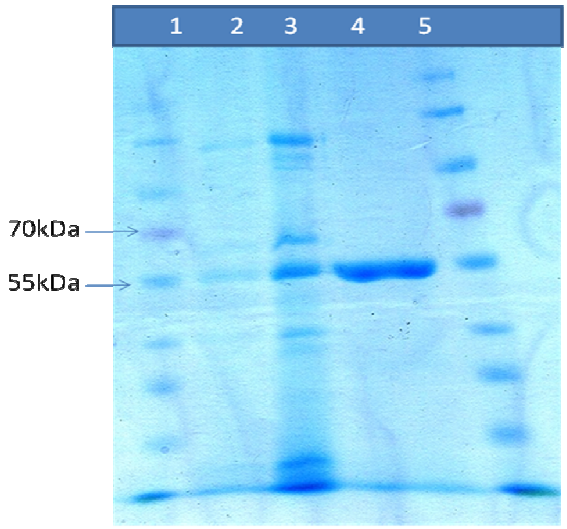


**Fig 2b. SDS-PAGE gel of purified recombinant cholesterol oxidase enzyme**
*Lane1, 5: Protein Marker, Lane 2, 3: Unpurified recombinant enzyme, Lane 4: Purified enzyme*

The presence of this band indicated that *CHO gene* has been correctly cloned. This finding was further confirmed by PCR assay and sequencing recombinant constructs (pET23a+CHO) (Fig.2a, 2b).

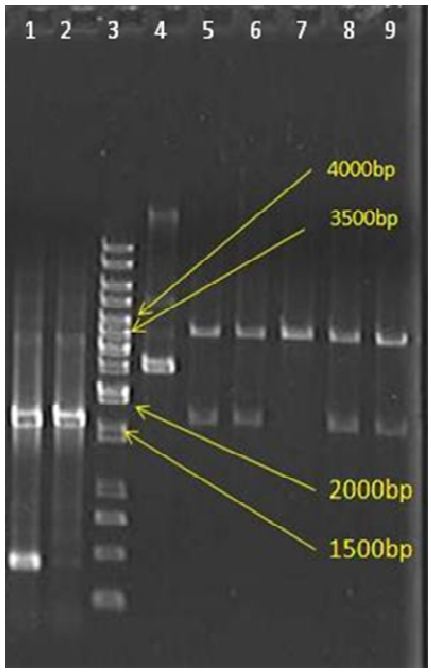


**Fig.3. Electrophoresis of PCR product resulting from cloning CHO gene into pET23a**
*Lane 1, 2: PCR product of CHO gene, Lane 3: 1 kb DNA ladder, Lane 4: pET23a,*
*Lane 5, 6, 8, 9: Digestion products of pET23a and CHO gene, Lane 7: Digestion of pET23a*

In enzyme activity measurement assay, supernatant of medium containing recombinant bacteria without IPTG inoculation and supernatant of recombinant bacteria under IPTG induction for 4 hour and crude extract of mentioned bacteria was separately examined for CHO activity. According to previous study, crude extract had more activity than supernatant (before and after IPTG inoculation) [10]. This finding indicating that CHO enzyme secretion is intra cellular form and requires particular methods for purification. Enzyme activity was assessed at 25 - 60 ˚C. The optimum temperature for highest activity of recombinant CHO enzyme was 45˚C (Fig.4). Recombinant CHO activity was also measured under different pH conditions (4.5 – 9.5) and in standard temperature (30˚C). It was shown that the most CHO activity is in pH = 7.5 (Fig. 5).

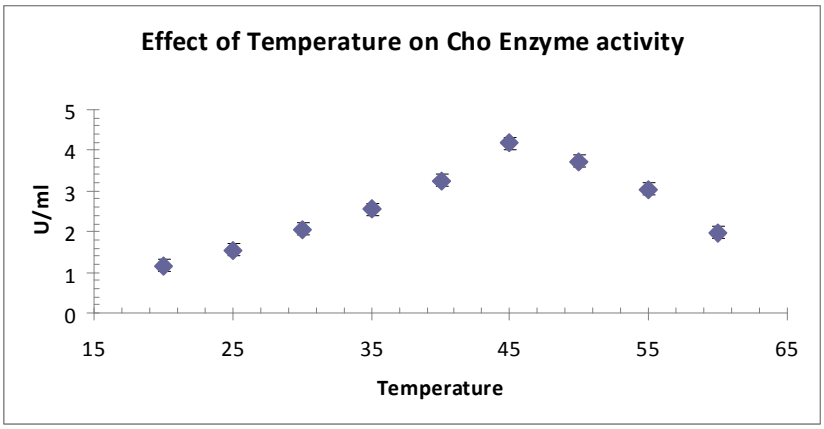


**Fig.4. Recombinant enzyme activity in different temperature**

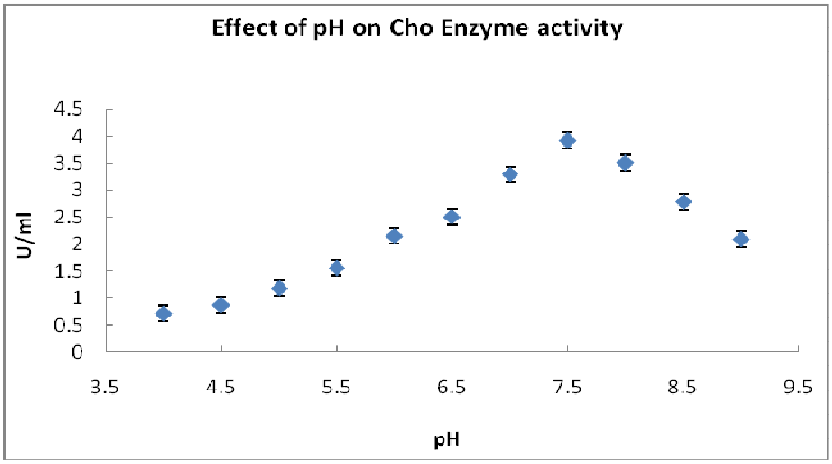


**Fig. 5. Recombinant enzyme activity in different pH**

Recombinant enzyme was stable in 45˚C and pH= 7.5 for one hour. Higher temperature and basic pH caused the enzyme to become unstable.

## DISCUSSION

Recently, cholesterol oxidase gene has been isolated from different microorganisms such as: *Brevibacterium Sterolicum*, *Arthrobacter simplex*, Streptomyces *sp. SA-COO*, *Rhodococcus Erythropolis*, *Burkholderia Cepacia* - 200, and their sequences have been determined for producing cholesterol oxidase [11,12]. Few investigations have been performed on cloning cholesterol oxidase gene of *Rhodococcus sp.* [13, 14]. In this study, *Rhodococcus sp.* isolated from Lorestan soil was selected for extraction of CHO gene. The gene has 1642 bp in length and encodes a protein containing 533 amino acids. Amino acid sequence of the gene was compared with the others and showed a high homology (96%) in gene bank. After cloning of CHO gene into *pET23a* expression vector, recombinant gene was expressed in *E coli BL21 plyss.* In order to increase the yield of active enzyme, the expression of recombinant gene was measured in optimum conditions for temperature, pH and IPTG concentration as inducer. Obtained results showed that the yield of active enzyme in temperature condition of 45˚C, pH=7.5 and 0.5 mM of IPTG concentration was about 4 U/ml. After protein expression, molecular weight of CHO recombinant enzyme was determined about 55 kDa using SDS-PAGE method which was in the same range of CHO with microbial source, (30-61 kDa). It was also similar to extra cellular enzyme of native *Rhodococcus* sp.501 [10]. Regarding to this note that pET vectors have strong promoter, a large amount of enzyme was secreted intracellular and a small of it was stored as inclusion body form which was inactive form of CHO enzyme. In this study, the optimum temperature condition for recombinant CHO activity was 45˚C that is in accordance with the other investigations (32-70˚C) [15]. In order to determine the stability of recombinant enzyme, enzyme activity in different temperature and pH conditions were evaluated for one hour. Resulting data showed that, recombinant enzyme kept its activity to 45˚C. At temperature conditions higher that 50˚C, enzyme activity would vigorously decreased. That was probably because of the destruction of enzyme structure at high temperature. Moreover, the CHO enzyme activity in different pH conditions was measured. Results demonstrated that recombinant enzyme was active in acidic to neutral pH for one hour. Basic pH causes enzyme activity to be decreased, seriously. Probably, they were more likely because of

chemical modification (deamination), hydrolysis of Asp-Pro linkages, and destruction of disulfide bonds and repulsion of charged amino acid residues.

Cholesterol oxidase enzyme obtained from Rhodococcus bacteria is one of the best and most accessible bacterial enzymes that is extracted and purified with the lowest cost. This enzyme has numerous medical and industrial applications.

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**Conflict of interest**
Authors have no conflict of interest in this study.

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