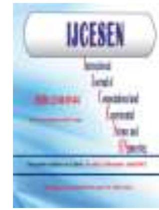
International Journal of Computational and Experimental Science and ENgineering (IJCESEN)

Vol. 8-No.3 (2022) pp. 69-73
http://dergipark.org.tr/en/pub/ijcesen



ISSN: 2149-9144

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Research Article

Heterologous Expression and Molecular Cloning from Williamsia Marianensis

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Article Info:

DOI: 10.22399/ijcesen.1133001
Received : 20 June 2022
Accepted : 29 September 2022

Keywords

Human infections
Williamson
PCR

Abstract:

The majority of therapy methods include downsides and limits. As a result, many researchers are focused on developing effective remedies. Therapeutic peptides, like proteins and antibodies, are a potential class of medications that have a number of advantages over traditional pharmaceuticals. Williamson marianensis-produced cholesterol oxididase has been demonstrated to have medicinal value. Using PCR and primers specific to an expression vector (pET28b), we were able to clone the cholestrol oxidase gene and express it in *E. coli* (BL-21/DE3) Rosetta following identification with IPTG. Genscript Corporation in the United States sequenced gyncholestrol oxidase (500 bp) to create a cox sequence, which was then submitted for synthesis. pET 28a(+) cox william showed a twofold restriction digestion pattern. The pattern was made up of two strands: one was a carrier plasmid (4200 bp) and the other was a 2800 base pair strand that contained the cholesterol oxidase gene. The cholesterol oxidase gene was successfully cloned and expressed as a consequence. Williamson marianensis-derived cholesterol oxidase will be exploited in future medicinal results.

1. Introduction

Human infections might be caused by the genus Williamsia [1], which belongs to the actinomycete family [2]. In the genus Williamsia, the DNA G+C content was 64–65 percent [3]. Williamsia infections and illnesses in people have been recorded, the illness was caused by exposure to the environment; however, no indication of an environmental source for Williamsia infections was found [4]. Marisch and his team were the first to describe W. muralis as the cause of lung infection in an elderly lady [5].
R. equi, a Gram-positive coccobacillus that dwells inside the host's macrophages and uses this enzyme as pathogenicity [6], was a potential pharmacological target for treating bacterial infections. Plasmids were also useful genetic tools for manipulating and analyzing microorganisms by introducing, modifying, or removing target genes [7].

There is no particular medium defined for the isolation of Williamsia from human clinical samples for identification by various culture media. Columbia agar supplemented with 5% sheep blood agar and brain heart infusion (BHI) agar [8], M3 agar supplemented with cycloheximide and nystatin [9], glucose/yeast extract agar (GYEA) plates [10], and raffinose [11].
Sequence-based identification was the most accurate approach and evaluation of taxonomic features for Williamsia identification. At the genus and species levels, 16S rRNA gene sequencing was an effective standard approach for accurately identifying new bacteria and emerging diseases [12]. Cholesterol oxidase is a bifunctional alcohol dehydrogenase/ oxidase flavoprotein that catalyzes the dehydrogenation of C(3)-OH in a cholestane environment to give the carbonyl product.

The interfacial catalysis association mechanism requires that the substrate binding site be directed toward and in contact with the lipid bilayer. The face that must be orientated towards the membrane holding the substrate was determined by X-ray crystal structures of the soluble enzyme in the absence of lipid [13]. These research focused on the Streptomyces and Rhodococcus equi enzymes because they were accessible to expression, mutagenesis, and higher solution crystallography. Their structures and mechanisms are almost similar [14].
The goal of this work was to identify and express the gene encoding cholesterol oxidase from Williamsia marianensis in E. coli, as well as to examine the sequence encoded cholesterol oxidase in silico.

1.1 Methodology

The activity of cholesterol oxidase was measured spectrophotometrically, as described before [15]. The hydrogen peroxide released by cholesterol oxidase's enzymatic digestion of cholesterol as a substrate might be detected by oxidative coupling of phenol 4-aminoantipyrine, catalyzed by horseradish peroxidase, in this approach. The latter reaction would produce quinoneimine red dye, which could be spectrophotometrically detected at 500 nm (Figure 1).

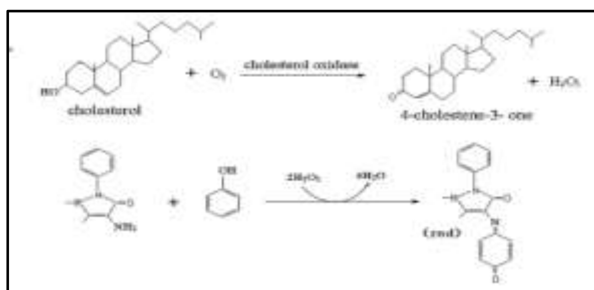

Figure 1. *Mechanism of cholesterol oxidase action on cholesterol as a substrate [16]*

The reaction mixture included 3 mM cholesterol in 1 mL 1 percent Triton X-100, 100 mL enzyme solution, 300 mM potassium phosphate buffer pH 7.0, 1.2 mM 4-aminoantipyrine, 21 mM phenol, and 20 U horseradish peroxidase, with a final volume of 3 mL. During the incubation time, the enzyme reaction was carried out at 37 °C for 10 minutes with moderate agitation. Boiling at 100°C for 3 minutes halted the reaction. Allow the reaction tubes to cool to ambient temperature. The generated color was then spectrophotometrically quantified at 500 milliseconds. The quantity of enzyme that released one micromole of H_2O_2 per minute at 37°C under the indicated test conditions was defined as one unit of enzymatic activity (U).

These primers were supplied in lyophilized form by (IDT/DNA Company), and the stock solution was prepared by dissolving the lyophilized primers in nuclease free water until a final concentration of 100 picomol/l, and the working solution was prepared by adding 10 l of stock solution to 90 L of nuclease free water to obtain a working primer solution of 10 picomol/l (stored at freezer -20 C).
Competent cells of the above-mentioned *E. coli* strain were produced using $CaCl_2$ as previously reported [17]. In a 250 mL Erlenmeyer flask, 100 mL LB was inoculated with 1 mL of overnight E. coli seed culture. The infected soup was incubated for 2-3 hours at 37°C with 200 rpm agitation until it reached an optical density of 0.4 at 600 nm. After that, the germs were put on ice for 30 minutes to cease growing. The cells were extracted by centrifugation at 4,500 rpm for 20 minutes at 4 degrees Celsius. The bacterial pellet was suspended in 20 mL of cool 0.1 M $CaCl_2$ and maintained on ice for 30 minutes after decanting the supernatant. Cells were harvested by centrifugation at 4,500 rpm for 20 minutes at 4°C. The bacterial pellet was suspended in 2 mL of chilled 0.1 M $CaCl_2$, and this bacterial suspension is chemically competent E. coli cells at this point.
Chemically competent E. coli cells were combined with 2-3L (50 ng) of the vector in an eppendorf tube (100L) (plasmid). For 40 minutes, the mixture was maintained on ice. The cells were then subjected to a heat shock in a water bath at 42°C for 45 seconds before immersing the eppendorf tube in ice for 5 minutes. The eppendorf tube was then filled with 900 mL of LB broth and incubated at 37°C for 1.5 hours with 180 rpm agitation. This 1 mL culture was disseminated on the surface of LB agar plates with the appropriate selectable marker kanamycin at a final concentration of 34 g/mL at the conclusion of the incubation period. The inoculated agar plates were then incubated for 24 hours at 37°C.

2. Results and Discussions

The map of the recombinant construct pET-28a (+)/Cox william, which was synthesized by GenScript Co. and created using SnapGene software, as shown in Figure 2. The recombinant plasmid is 7045 bp long after the cholesterol oxidase gene has been inserted. The whole length is 7045 bp, as displayed. In terms of nucleotide and protein molecular weights, the predicted recombinant protein cholesterol oxidase should be 1821bp and 69.3 kDa, respectively. The cholesterol oxidase gene was 1821bp in length. The 6-His tag would provide the recombinant cholesterol oxidase protein an additional 18 amino acids. Furthermore, the XhoI recognition site would add two additional amino acids.

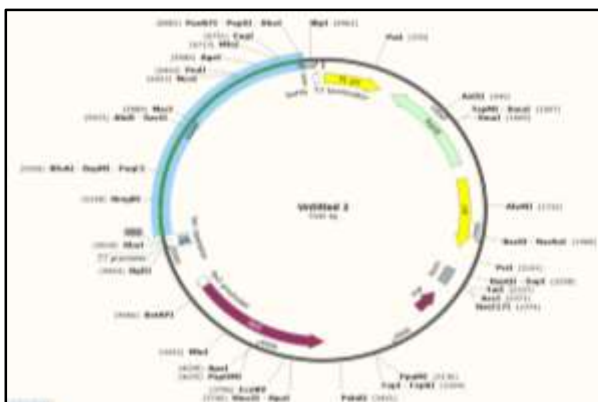

Figure 2. The map of the recombinant construct pET-28a (+)/Cox_william

As a consequence, 23 more amino acids would be added to the recombinant cholesterol oxidase protein, resulting in the creation of a 630 amino acid recombinant cholesterol oxidase protein. The final recombinant protein would have had 629 amino acids when the stop codon was removed. The recombinant protein's anticipated molecular weight is 69.3 kDa. The recombinant Williamsia marianensis strain of the Cox william gene was expressed in *E. coli* BL21 (DE3) Rosetta Strain; host strain detects an able level of gene expression was achieved after induction with (one mM of IPTG), for 18 hours at 30°C; then after cell lysis, the protein extract (soluble fraction) of the recombinant protein was purified with IMAC Chromatography. Figure 3 shows the twofold restriction digestion pattern of pET-28a (+)/Cox William. Two bands were obtained: one for the plasmid vector (4200 bp) and another for the cholesterol oxidase gene (2800 bp).

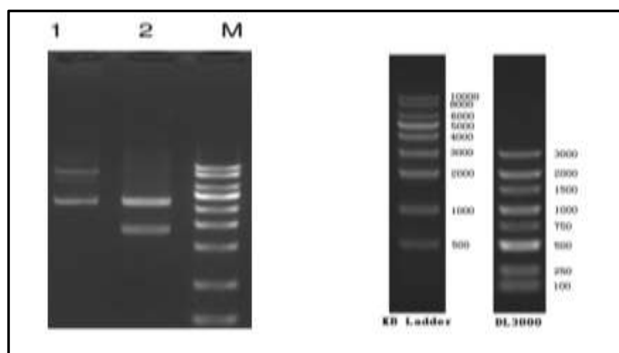

Figure 3. Agarose gel electrophoresis (1%) of restriction pattern digestion of pET-28a (+)/Cox_william with MluI and XhoI

Prokaryotic microbes created all of the cholesterol oxidase (pathogenic and nonpathogenic bacteria). Nonpathogenic bacteria use ChO as a metabolic tool for getting carbon sources from cholesterol breakdown, but pathogenic bacteria use it to infect host macrophages by oxidizing membrane cholesterol [18]. Many attempts have been attempted to extract ChO from original microorganisms so far. However, there are several drawbacks to this strategy, such as challenging growing circumstances and limited production of the original microorganisms [19]. ChO genes from several bacterial sources have been cloned and expressed, indicating that they are suitable for commercial enzyme production [20].

These "recombinant" forms were employed to effectively convert *E. coli*; the process seemed to produce 100% recombinant clones. (Shuldiner and his friends devised another LIC approach that utilizes denaturation and heterologous annealing of the PCR product and vector and is difficult to regulate [21]. The automodel command was then used to conduct homology modeling. The variable target function approach with conjugate gradients was then used to improve each model [22]. The current work produced a phylogenetic tree based on detected nucleic acid variations. This tree depicted the genetic relationship between Williamsia marianensis cholesterol oxidase and cholesterol oxidases from other species.

3. Conclusions

The purpose of this study was to find and express the cholesterol oxidase gene from Williamsia marianensis in E. coli, as well as to look at the sequence encoded cholesterol oxidase in silico. The findings of this investigation revealed the three-dimensional structure of Williamensia marienesi cholesterol oxidase protein sequence. Burkholderia cepacia FAD glucose dehydrogenase gamma-alpha subunit complex crystal structure).Improved enzymatic performance had been attained using a design based on structure and function relationships. Qin and his team in 2014 were investigated substrate selectivity and affinity by introducing amino acid alterations into Streptomyces cholesterol oxidase utilizing site-directed mutagenesis and structural comparisons [22].

The recombinant Cox william construct was first expressed at 4.5 U/mL in E. coli BL21 (DE3) Rosetta strains bearing the pET-28a (+)/Cox william construct. When the LB growing medium was replaced with the applied optimized method, the traceable Cox william construct activity in the cell lysate of the recombinant Rosetta strain was effectively increased. After ion exchange column separation, cholesterol oxidase was purified to homogeneity and contained DEAE Sepharose CL-6B with 501.05 U total activity and 31.15 mg protein. With a specific activity of 16.08 U/mg of protein, the enzyme was isolated [23]. In *E. coli* BL21 (DE3) Rosetta strains carrying the pET-28a (+)/Cox william construct, the recombinant Cox william construct was initially produced at 4.5 U/mL. The traceable Cox william construct activity in the cell lysate of the recombinant Rosetta strain was effectively raised

when the LB growth medium was changed with the applied optimized technique.

Author Statements:

- **Ethical approval:** The conducted research is not related to either human or animal use.
- **Conflict of interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper
- **Acknowledgement:** The authors declare that they have nobody or no-company to acknowledge.
- **Author contributions:** The authors declare that they have equal right on this paper.
- **Funding information:** The authors declare that there is no funding to be acknowledged.
- **Data availability statement:** The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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