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Molecular characterization and chemical engineering of cholesterol oxidase from endophytic bacteria in some medicinal plants

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***ABSTRACT:** Cholesterol oxidase (COX) is an essential enzyme involved in cholesterol oxidation, playing a vital role in various biochemical processes. This study focused on the molecular characterization and chemical engineering of COX derived from endophytic bacteria found in medicinal plants. The COX gene in these bacterial strains was successfully amplified and characterized using molecular methods such as polymerase chain reaction (PCR) and DNA sequencing. Analysis of the obtained sequences provided insights into the genetic diversity and phylogenetic relationships of the COX gene in these bacteria. The enzymatic properties of the purified COX enzyme, including substrate specificity, optimal conditions, and stability, were also investigated. These findings enhance our understanding of the catalytic potential and functional features of COX from endophytic bacteria in medicinal plants. Additionally, the study explored chemical engineering approaches to improve the enzymatic efficiency and stability of COX. Strategies like enzyme immobilization and protein engineering were employed to enhance the performance of COX. This chemical engineering aspect offers opportunities for the development of more efficient and robust COX-based biocatalysts. The molecular characterization and chemical engineering of COX from endophytic bacteria found in medicinal plants hold significant value. The insights gained not only expand our knowledge of the genetic diversity and enzymatic properties of COX but also lay the groundwork for potential applications in biotechnology and the pharmaceutical industry. Harnessing the catalytic capacity of endophytic bacteria's COX may lead to the creation of innovative therapeutic drugs and diagnostic tools for cholesterol-related disorders.*

***KEYWORDS:** Oxidoreductase enzymes. cholesterol oxidase, molecular characterization, Biotechnological applications, Endophytic bacteria*

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I. INTRODUCTION

Cholesterol oxidase is an enzyme that converts cholesterol to cholest-4-en-3-one. and hydrogen peroxide. **(Akanksha *et al.*, 2020** and **Vasanthakumar and Kuppusamy 2022**). Several bacterial species, including *Streptomyces* and *Rhodococcus* sp., manufacture it. The enzyme is widely employed in a wide range of applications, including medical diagnostics, biotechnology, and the food sector. It is frequently used to detect cholesterol in blood serum, food, and other biological substances (**Rani and Chandel, 2019; Puri *et al.*, 2021).** Cholesterol oxidase has a molecular weight of 50-75 kDa and is best active at neutral to slightly alkaline pH levels and moderate temperatures (30-45oC). The flavoprotein contains a flavin adenine dinucleotide**.** (FAD) cofactor that is essential for catalysis. Cholesterol oxidase's structural and functional features, including substrate specificity, kinetics, and mechanism of action, have been thoroughly explored. **(Zhang *et al.*, 2020; Torkaman and Soudi 2021).**

1- Endophytic bacteria is a promising source of bioactive compound

Endophytic bacteria have been identified as a new source of bioactive chemicals (**Singh *et al.*, 2017a and 2017b**). They are bacteria that dwell within plant tissues without inflicting any harm to the plant. They colonize the intercellular gaps of plant cell walls and xylem vessels, as well as tissues of flowers **(Compant *et al* 2011 and Zheng *et al*.2022).** Cholesterol oxidase is a commercially valuable enzyme that is extensively employed in laboratories that regularly measure cholesterol levels in food, blood, and clinical samples. **(Fazaeli *et al.*, 2018**). The researchers created a quick and sensitive cholesterol assay using recombinant cholesterol oxidase in this work. Affinity chromatography was used to purify the enzyme after it was expressed in E. coli. The technique demonstrated great specificity and sensitivity for detecting cholesterol in serum and food samples. **Ghosh *et al.***

(2018). Also conducted research on a colorimetric biosensor based on cholesterol oxidase for the fast detection of cholesterol in serum. The researchers developed a colorimetric biosensor based on cholesterol oxidase for the rapid detection of cholesterol in serum. The biosensor was built by immobilizing the enzyme on a gold electrode surface and demonstrated good sensitivity and selectivity for detecting cholesterol. Cholesterol oxidase is extensively used in clinical and commercial settings to evaluate cholesterol concentration for arteriosclerosis diagnosis and to analyse steroids in food samples. and creating 3-ketosteroids from corresponding steroids **(kumari *et al.*, 2012).** Furthermore, cholesterol oxidase has been identified as a promising natural anticancer medication **(El-Naggar *et al.*, 2018**).

2- Role of cholesterol oxidase from endophytic bacteria in the metabolism of cholesterol:

Cholesterol oxidase is an enzyme that is crucial in the metabolism of cholesterol. Cholesterol is a lipid molecule that is required for the proper operation of the body's cells.; nevertheless, elevated levels of cholesterol in the bloodstream can cause atherosclerosis, a condition in which plaque builds up on the walls of arteries, leading to heart disease and stroke. Endophytic bacteria may metabolise cholesterol and utilise it as a carbon or energy source by employing the extracted cholesterol oxidase in the early step of cholesterol metabolism. **Kanwar and Davi (2017).** Cholesterol oxidase destroys sterols at the 3-hydroxyl sites, producing 4-cholesten-3-one and H2O2. The initial stage in the microbiological breakdown of cholesterol is the oxidation of the 3-hydroxyl group by cholesterol oxidase.. (**Bokoch *et al.*, 2004; Vasanthakumar and Kuppusamy 2022).** The steroid moiety is finally broken down, yielding carbon dioxide and water as byproducts. (**Kumari and Kanwar, 2016).** Cholesterol oxidase's catalytic mechanism has been widely explored utilising a combination of kinetic, spectroscopic, and structural approaches. These investigations shed light on the impact of the FAD cofactor and the protein environment in modifying the enzyme's reactivity and specificity. The principal function of the enzyme is to catalyse the oxidation of cholesterol to cholest-4-en-3-one, which is catalysed by the enzyme's fad cofactor. As a consequence of the transfer of two electrons from cholesterol to molecular oxygen, hydrogen peroxide is produced.

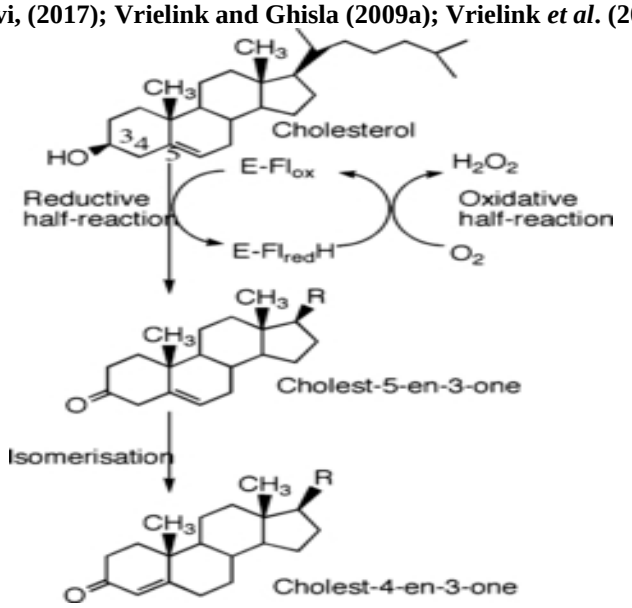
Many bacterial species have been implicated in cholesterol biodegradation via bifunctional, flavin adenine dinucleotide (FAD)-containing cholesterol oxidase, which oxidises the cholesterol and produces 4-cholesten-3-one with oxygen reduction to hydrogen peroxide (**Lashgarian et al., 2016**

Cholesterol oxidase is a monomeric enzyme that catalyses two stages in the conversion of cholesterol to cholest-4-en-3-one: The first is that cholesterol is oxidised to cholesterol -5 and 3-. The second is that the cholest-5-en 3 isomerization reaction may be reversible (**Ahn and Sampson, 2004; Lim et al., 2006**). Motteran and colleagues (2001) found, however, that the intermediate 3-ketosteroid isomerization step leads to the final product cholest -4-en -3-one. .

$$O2 + \text{Cholesterol} \rightarrow H2O2 + \text{Cholest -5-en-3-one}$$

$$\text{Cholest -5-en -3-1} \rightarrow \text{Cholest -4-en -3-one}$$

[Kanwar and Davi, (2017); Vrielink and Ghisla (2009a); Vrielink *et al*. (2010); Fazaeli *et al*., (2018)]



Scheme 1. The reactions catalysed by cholesterol oxidase and the structures of the species involved. The terms reductive and oxidative half-reactions refer to redox state changes in the bound flavin coenzyme. (**Kanuer and Davi, 2017)**

Cholesterol oxidase catalyses three chemical reactions (Scheme 1). The two redox equivalents created are transferred to the (oxidised) flavin cofactor, which is reduced in the process. In the second catalytic step, the reduced flavin interacts with dioxygen to regenerate the oxidised enzyme and hydrogen peroxide (H2O2). (oxidative half-reaction). In the third catalytic step, the oxidised steroid isomerizes the double bond in the steroid ring system from D5-6 to D4-5 to produce cholest-4-en-3-one. This isomerization process occurs more quickly than the enzyme's release of the intermediate, cholest-5-en-3-one. Several studies have been undertaken to study the specificity of cholesterol oxidase for various substrates derived from the cholestane skeleton, notably those reporting recently discovered cholesterol oxidase **(Kreit and Sampson 2009**). While the dehydrogenation of the CH-OH function at position 3 of cholestane is conserved, the addition of functional groups that alter the polarity and hydrophobicity of the same has deleterious consequences (**Pollegioni *et al*.,1999)**. The oxidation of cholesterol by cholesterol oxidase is a crucial step in the metabolism of this lipid molecule by microbes, and it has various potential applications in biotechnology, medicine, and food science, according to **Wali *et al*. (2019).** The goal of this enzyme article is to critically analyze and summarize the findings of a research paper that investigates the molecular and biochemical mechanisms that cause cholesterol oxidase to be purified, as well as the methodologies used to characterize its molecular structure and function. Furthermore, the evaluation may describe the enzyme's prospective application in other areas such as food, paratheatrical, and biotechnology.

3- Endophytic microorganisms- producing cholesterol oxidase

Endophytic bacteria are microorganisms that stay inside plant tissues without harming the plant. These bacteria can aid in the growth of the plant and protect it against disease. *Bacillus citrus* is one of numerous endophytic bacteria that have been identified to manufacture cholesterol oxidase by **Khandelwal *et al* (2015). Wali *et al*. (2019)** discovered cholesterol degradation in Gram-positive bacteria such as *Myobacterium*, *Rhodococcus*, *Brevibacterium*, *Streptomyces*, and others, as well as Gram-negative taxa such as *Comamonas*, *Burkholderia*, *Pseudomonas*, and *Chromobacterium*. **Abd El-wahed *et al*., (2021)** isolated and identified cholesterol oxidase generating *Actenomycetes* from Egyptian soil for large-scale fermentation in submerged cultures. The study focusing on the spot of cholesterol oxidase generating genera of bacteria from previous studies were summarized in table (1), and it could be inferred that the extracellular bacterial cholesterol oxidase has garnered significant attention by many reserchers.

Table 1: List of some microbial genera-producing cholesterol oxidase

Microorganisms	Reference
Actenimycetes sp.	**Abd El-wahed *et al*., (2021)**
Lavendulae mycelium	**Petrova *et al*., (1979)**
Arthrobacter	**Chen *et al*., (2006)**
Arthrobacter simplex	**Liu *et al*., (1988)**
Bacillus cereus	**Vasanthakumar and Kuppusamy (2022)**
Bacillus cereus KAVK4	**Kuppusamy and Kumar, (2016)**
Bacillus pumilu and Serrat marcescens	**Wali *et al*., (2019)**
Bacillus spp.	**Rhee *et al*., (2002)**
Bordetella sp.	**Lin *et al*., (2010)**
Brevibacterium	**Uwajima *et al*., (1973)**
Brevibacterium sterolium	**Croteau and Vrielink., (1996)**
Brevibacterium sterolium	**Fujishiro *et al*., (1990)**
Brevibacterium sterolium	**Motteran *et al*., (2001)**
Castellaniella *sp*	**Devi *et al*., (2021)**
Chromobacterium	**Doukyu et al., (2008)**
Chryseobacterium gleum	**Reiss *et al*., (2014)**
Corynebacterium	**Shirokane and Mizusawa, (1977)**
E. coli strain	**Fazaeli *et al*., (2019)**
Enterococcus hirae	**Yehia *et al*., (2015)**
Gamma proteobacterium Y-134	**Isobe *et al*., (2003)**
Lactobacillus sp.	**Kulkarni *et al*., (2013)**
Micrococcus sp	**Kanchana *et al*., (2011)**

Mycobacterium	**Brzostek *et al.*, (2007)**
Nocardia erythropolis	**Shirokane *et al.*, (1977)**
Nocardia rhodochrous	**Cheetham *et al.*, (1982)**
Pseudomonas	**Lee *et al.*, (1989)**
Pseudomonas sp	**Doukyu and Aono, (1998)**
Rhodococcus	**Yazdi *et al.*, (2008)**
Rhodococcus equi	**Shi and Peng (2005)**
Rhodococcus sp.	**Lashkarian *et al.*, 2010)**
Rhodococcusequi	**Bokoch *et al.*, (2004)**
Streptomyces aegyptia	**El-Naggar *et al.*, (2018)**
Streptomyces cavourensis	**El-Naggar *et al.*, (2016)**
Streptomyces fradiae	**Yazdi *et al.*, (2001b)**
Streptomyces griseocarneus	**Sampson *et al.*, (2003)**
Streptomyces rochei	**Elsayed and Abdelwahed (2020)**
Streptomyces sp	**Praveen *et al.*, (2011)**
Streptoverticillium cholesterolicum	**Inouye *et al.*, (1982)**

4- Production of cholesterol oxidase from endophytic species and its culture conditions

Because of their potential uses in fields such as agriculture, medicine, and biotechnology, endophytic bacteria have been the subject of several scientific studies (**Siddalingeshwara et al., 2013; Kumar et al., 2014).**

The production process and culture conditions necessary for efficient cholesterol oxidase production from endophytic species **(Pandy *et al* (2000):**

1- The first step in cholesterol oxidase production is the selection and isolation of endophytic species capable of producing the enzyme. Various medicinal plants can be explored to identify endophytic microorganisms with cholesterol oxidase-producing potential. Isolation techniques such as surface sterilization, serial dilution, and plating on selective media can be employed to isolate the desired endophytic species.

2- Screening for cholesterol oxidase-Producing Strains: cholesterol oxidase After isolating the endophytic species, screening methods are employed to identify strains that exhibit cholesterol oxidase production. Screening can be done qualitatively by observing the formation of a characteristic yellow halo around colonies on cholesterol-containing agar plates. Quantitative screening can be performed by assaying the enzyme activity of the isolated strains.

3- Optimization of Culture Conditions: Once -producing strains are identified, the culture conditions need to be optimized to enhance enzyme production. Factors such as temperature, pH, carbon and nitrogen sources, aeration, and agitation play crucial roles in the growth and production of cholesterol oxidase. [**Khan and Akhtar (2015) & Singh and Kumar, (2019)]**

i. Temperature and pH: The optimal temperature and pH range for cholesterol oxidase production should be determined through a series of experiments. It is essential to maintain the culture conditions within the suitable range to maximize enzyme production.

ii. ii. Carbon and Nitrogen Sources: The carbon and nitrogen sources used in the culture medium have a big influence on cholesterol oxidase synthesis. Various carbon sources, such as glucose, glycerol, or starch, as well as nitrogen sources such as peptone, yeast extract, or ammonium salts, can be examined.

iii. Aeration and Agitation: Proper aeration and agitation are crucial for the growth and productivity of cholesterol oxidase-producing cultures. It ensures sufficient oxygen supply and prevents the formation of oxygen gradients within the culture medium.

iv. Fermentation Process: Fermentation techniques, such as submerged fermentation or solid-state fermentation, can be employed for cholesterol oxidase production. The appropriate fermentation process should be selected based on the characteristics of the endophytic species and the desired enzyme yield

v. Downstream Processing and Enzyme Extraction: After successful cholesterol oxidase production, downstream processing techniques, such as cell harvesting, cell disruption, and protein extraction, are employed to obtain the purified enzyme. Methods like sonication, high-pressure homogenization, or enzyme release agents can be used for cell disruption,

followed by centrifugation or filtration to separate the cell debris from the enzyme extract. **Srivastava *et al.* (2021)** isolated a cholesterol oxidase-producing endophytic bacterium from medicinal plant leaves. The bacteria was identified as *Bacillus velezensis* based on the 16S rRNA gene sequence. Other researchers purified and characterized an endophytic Streptomyces sp. cholesterol oxidase. This study's findings shed light on the potential biotechnological and medical applications of cholesterol oxidase generated by endophytic bacteria **(Biswas *et al.*, 2021).**

Endophytic bacteria were cultivated in various modified media to study the production of cholesterol oxidase. Bacterial cells were cultivated at 30 ^{0}C with 150 rev min-1 shaking. Another Media, with a pH of 7, comprises 1.5 potato starch, 0.4 yeast extract, 0.2 malt extract, 0.5 peptone, 0.1 K2HPO4, 0.05 MgSO4.5H2O, and 0.05 NaCl. *Pseudomonas aeruginosa* and Bacillus citrus were grown in a mixture containing 0.1% NH4NO3, 0.025% KH2PO4, 0.025% MgSO4, 7H2O, 0.0001% Fe SO4, 0.5% yeast extract, 0.2% cholesterol, and 0.3% (v/v) 80 by **Khandelwal *et al.*, 2015** and **Wu *et al.*, 2015.** According to **Kuppusamy and Kumar (2016),** the *Bacillus cereus* bacterial species was cultivated in cholesterol-containing nutrition medium and dox media at 37°C for 24 hours.

Other media included 050 g/litre KH2PO4, 025 g/litre Na2HPO4, 010 g/litre L-valine, 015 g/litre MgSO47H2O, 001 g/litre ZnSO47H2O, 010 g/litre FeSO47H2O, and 40 ml/litre Tween 80. Last liquid medium were used by **Fazaeli *et al.*, (2019)** for *Chromobacterium strain* was Luria-Bertani (LB, 10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, Merck), Super Broth (SB, 32 g/L peptone, 20 g/L yeast extract and 5 g/L NaCl, Merck), Terrific Broth (TB, 12 g/L peptone, 24 g/L yeast extract, 8 g/L glycerol, 17 mM KH2PO4 and 72 mM K2HPO4, Merck). **El-Sayed and Abd el-wahed (2020**) revealed *Streptomyces rochei NAM-19*, a novel strain obtained from soil and exhibiting medium optimisation utilising Response Surface Methodology for improved cholesterol oxidase production.

Pollegioni *et al*. (2009) Investigate how cholesterol oxidase produces H2O2, which is then examined enzymatically by horseradish peroxidase via the oxidative reaction of 4-aminoantipyrine and phenol, generating a quinoneimine dye at absorption 500nm. However, assessing bile cholesterol is difficult due to pigm Streptomyces rochei NAM-19, a novel strain isolated from soil, and dient interference; thus, an electrochemical method based on oxygen consumption by cholesterol oxidase reaction is used. **Cheillan and colleagues (1989).** Another culture environment, such as substrates, altered cholesterol oxidase synthesis from various bacterial sources. The type of substrate utilized in the fermentation process can influence bacterial production of cholesterol oxidase. Substrates that have been found to stimulate cholesterol oxidase production by bacteria include glycerol addition in the fermentation medium, significantly boosted cholesterol oxidase synthesis by the bacteria *Streptomyces sp. S27*. The ideal glycerol concentration was found to be 3% (v/v), This resulted in a 3.2-fold increase in cholesterol oxidase synthesis when compared to the control **(Zhang *et al.*, 2014**). Another study used the fermentation medium to stimulate the bacterium *Rhodococcus sp. AS2's* manufacture of cholesterol oxidase. (**Li *et al.*, 2015**). Casien was also reported by **Liu *et al.* (2017a**) as an inducer of cholesterol oxidase synthesis by *Streptomyces sp. CH7*. Supplementing the fermentation media with peptone and potato starch increases cholesterol oxidase production in Bacillus sp. KJ-1 and *Streptomyces sp. S9*. **(Zhang *et al.*, 2019**).

Under optimal growth conditions, *E. hirae Mil-31* isolated from milk has a significant capacity to deconstruct cholesterol in basal medium supplemented with cholesterol, and the decomposition process of cholesterol by this strain is caused by the formation of cholesterol oxidase enzyme (**Yehia *et al.*, 2015**). *Streptomyces sp., Arthrobacter sp., and Aspergillus sp.* were identified as the bacteria producing cholesterol oxidase in oil mill waste, dirt, and compost (Parekh and Desai, 2012). Other Gram-positive and Gram-negative bacteria that have received a lot of interest are Comamonas, *Burkholderia, and Enterococcus*. **(Vrielink and Ghisla, 2009; Yehia et al., 2015).** Some more Gram-positive and Gram-negative genera include *Enterococcus, Pseudomonas, Chromobacterium* sp., *Bacillus pumilu, and Serratia marcescens* **(Wali *et al.*, (2019)** and *Streptomyces rocheiNAM-19 Strain* **(Elsayed and Abd el-wahed, 2020)**.

5- Purification of cholesterol oxidase from endophytic bacteria:

Cholesterol oxidase is an enzyme that is essential in the oxidation of steroids. purification from endophytic bacteria has recently been studied. The purification process involves identifying the gene that encodes the protein, expressing the gene in a suitable host system, and then purifying and characterizing the protein using various techniques, including chromatography, SDS-PAGE, Western blotting, and enzyme activity assays. (**Dholakiya & Patel (2019)**. Studies have explored various methods for improving the yield and purity of cholesterol oxidase, including the use of different chromatography techniques, optimization of culture conditions, In addition, surfactants were added to the medium. **Gao et al. (2020)** employed ion exchange and gel filtration chromatography to purify and characterize cholesterol oxidase from Streptomyces griseus. The isolated enzyme demonstrated good activity and stability throughout a wide pH and temperature range. They also looked at how various surfactants influenced the synthesis and purification of Streptomyces sp. cholesterol oxidase. Tween 80 and Triton X-100, on the other hand, significantly increased enzyme activity in the culture medium.. The addition of SDS inhibited it. Surfactants were also used to increase the yield and purity of the purified enzyme.

Certainly! Here are some key points regarding the purification of cholesterol oxidase (COX) from endophytic bacteria, supported by general information:

1- Crude Extract Preparation: The purification process usually begins with the preparation of a crude extract containing COX. This involves culturing the endophytic bacteria under optimized conditions and harvesting the biomass.

2- Cell Disruption: The harvested cells are subjected to cell disruption methods to release the intracellular components, including COX. Techniques such as sonication, high-pressure homogenization, or enzyme release agents can be employed to break open the cells and release the cellular contents.

3- Removal of Cell Debris: The cell debris is separated from the crude extract using centrifugation or filtration techniques. This step helps eliminate unwanted insoluble cellular material and obtain a clarified extract.

4- Protein Precipitation: The clarified extract can be subjected to protein precipitation methods such as ammonium sulfate precipitation or organic solvent precipitation. These methods help in concentrating the COX and removing other proteins present in the extract

5- Chromatographic Techniques: For COX purification, several chromatographic techniques such as affinity chromatography, ion exchange chromatography, or size exclusion chromatography are typically utilised.. These techniques exploit the differences in physical and chemical properties of proteins to separate and purify COX from other contaminants.

6- Activity Assays: Throughout the purification process, it is essential to monitor COX activity to assess the enzyme's purification progress. Activity assays, typically based on the measurement of substrate conversion or product formation, are performed to determine the specific activity of the enzyme.

7- Dialysis and kinetic parameters, and stability profiles. Concentration: Following purification, the COX fractions are often subjected to dialysis to remove unwanted salts and buffer components. Concentration techniques like ultrafiltration or lyophilization may also be employed to obtain a purified and concentrated enzyme.

8- Enzyme Characterization: After purification, the purified COX can be characterized for its biochemical and enzymatic properties, including substrate specificity, molecular weight determination, pH and temperature optima,

Table (2): list of some purified methods for cholesterol oxidase

Purification methods	Purification fold	Yield	Specific activity U/mg	Reference
Ammonium sulfate precipitati 40 -70% saturation and dialyses	18.8	30%	0.9	**Bhattacharyya ar Ghosh (1975)**
(DEAE-cellulose column elut with a linear NaCl gradient) Ic exchange chromatography.	45	40%	2.2	**Puri and Yady (2007)** **EL-sayed *et al* (2019)**
Ion exchange chromatography	21	62%	2.5	**El-sayed and Ali (2011)** **Gao *et al.*, (2020)**
Affinity chromatography (Ce free extract was applied cholesterol-BSA-Sepharose column eluted with cholesterol solutio	51	28%	5.3	**Singh *et al.*, (2016)**
Affinity chromatography	34 28	82% 63%	3.5	**Zarei *et al.*, (2012)**
Ultrafiltration and gel filtrati with Sephadex G-100 column	19	55%	7.9	**Kadmy and Farha (2014)**
Hydrophobic interacti chromatography	71	60%	22.3	**Lee *et al* (2006)**
Two-step purification using io exchange and hydrophobic interaction chromatography	109	10%	9.8	**Zhang *et al.*, (2018)**
Affinity chromatograp (cholesterol-agarose column, t protein was eluted with 0.1 NaOH.	42	43%	5.6 2.5	**Singh *et al.*, (2011) Zar *et al.*, (2022)**
Ion exchange chromatography	57 43.6	32% 47.2%	6.2 9.8	**Soudi *et al.*, 2023** **Lee *et al.*, 2012**

(**Fujishiro *et al.*, 2002; Li *et al.*, 2019; Chen *et al.*, 2021)** also investigated different purification methods for CO enzyme, including affinity chromatography, ion exchange chromatography, and ultrafiltration. The purified enzyme demonstrated great activity and stability throughout a wide pH and temperature range, making it suitable for the detection of cholesterol in dietary samples. Other studies have focused on increasing the yield and purity of cholesterol oxidase from endophytic bacteria using a variety of techniques, such as different chromatography techniques, optimization of culture conditions, and the addition of surfactants to the medium (**Li *et al.*, 2021**). These findings have significant implications for the use of cholesterol oxidase in a variety of biotechnological applications, including the detection of cholesterol in dietary samples and the development of novel steroid transformation therapy techniques. These methods provide improved specificity and yields of pure cholesterol oxidase enzyme, making them promising for industrial and medical applications **Elsayed *et al* (2019)**.

6- Physicochemical properties of endophytic microbial cholesterol oxidase

Cholesterol oxidase production from endophytic organisms necessitates rigorous strain selection and isolation, Then, the culture conditions are optimized. Temperature, pH, carbon and nitrogen supply, and aeration, and agitation must all be optimized to maximize cholesterol oxidase production. The fermentation process and downstream processing procedures used are critical to producing the pure enzyme. By focusing on these factors, it is possible to achieve effective cholesterol oxidase production from endophytic species for a variety of applications in diagnostics, treatments, and biotechnological processes. The physical parameters of cholesterol oxidase from various microorganisms are detailed in **Table (3),** including molecular weight, ideal pH and temperature for enzyme activity, and specific activity in U/mg protein. For various bacteria, the ideal pH ranged from 5.0 to 9.0, and for all enzymes, the ideal temperature range fell between 25 and 65 degrees Celsius. According to several

sources, the enzyme's open reading frame encoded 389-395 amino acids with anticipated molecular weights of 46.0-62.0 kDa for each subunit. Previous study demonstrated the optimal pH and temperature for cholesterol oxidase activity and stability, as well as the fact that the enzyme had the same conformational shape in diverse bacterial species. (**Doukyu *et al.*, 2008; Saranya *et al.*, 2014; El-Naggar *et al.*, 2017**).

Table (3). Physicochemical properties for cholesterol oxidase from different endophytic bacterial species

Microbes	Mol. wt. (kDa/subunit)	optima pH	Opt. Temp (°C)	Specific activity U/mg protien	References
Castellaniella sp	59	8	40	15	Devi *et a* 2021
Chromobact-erium sp.	58	7	70	4.6	Fazaeli *et a* 2019
E. hirae Mil-31	60	7.8	40	124	Yehia *et a* 2015
Marine Streptomyces sp.	42-84	9	60	1.22	Savithri *et a* 2019
Bacillus subtilis	105	7.5	37	1.39	Kumari a Kanwar (2016)
Bordetella sp	55	7.0	37	20.8	Lin *et a* (2010)
Pseudomonas aeruginosa	59	70	60	11.6	Doukyu a Nihei, (2015)
Rhodococcus sp	60	8.0	37	35.64	Kasabe *et a* (2015)
Y-Proteobacterium	58	6.5	50	14.4	Isobe *et a* (2003)
Arthrobacter simplex	57	7.5	50	3.6	Doukyu *et a* (2008)
Streptomyces parvus	55	7.2	55	20	Praveen *et a* (2011)
Chryseobacterium gleum	60	6.72	37	15.5	Reiss *et a* (2014)

Wang *et al.* (2008) found that metal ions such as silver and mercury compounds significantly inhibited cholesterol oxidase activity, Glutathione, In contrast, p-chloromercuric benzoate, mercuric chloride, or silver nitrate nearly totally eliminated inhibition. These data show that SH groups may play a role in cholesterol oxidase catalysis. Suicide inhibitors are utilised in "rational drug design," which aims to generate a novel substrate based on previously established processes and substrates. The major goal of this method is to develop substrates that are unreactive until they reach the active site of an enzyme while still being highly selective. This strategy has resulted in drugs with very little side effects **(Johnson *et al*, 2010).** Morpholine derivatives, particularly fenpropimorph and tridemorph, were discovered to preferentially inhibit the isomerization activity of *Nocardia erythropolis* cholesterol oxidases **(Hesselink *et al.*, 1990; Devi and Kanwar, 2017). Yazdi *et* al. (2001a)** investigated the impacts of several surfactants, finding that Tween 80 and Tween 60 had the greatest effect on cholesterol production, while Triton X100 nearly prevented enzyme production. **Moradpour *et al.* (2013)** discovered that following medium Tween 20 supplementation, *Streptomyces badius* produced much more cholesterol oxidase. Unlike **Doukyu *et al.* (2008),** detergents decreased CHOx production in *Nocardia erythropolis and Pseudomonas sp.* According to **MacLachlan *et al.*, (2000) and Doukyu *et al.*, (2001),** Fenpropimorph:6 50 mg/l, 50% inhibition, and Sarkosyl:12 1%, 56% inhibition.

7- Crystal structure of cholesterol oxidase

Cholesterol oxidase is a flavoenzyme that catalyses cholesterol oxidation and isomerization to cholest-4-en-3-one. X-ray crystallography was used to establish the structural structure. Cholesterol oxidase's crystal structure reveals a homodimer protein with a flavin adenine dinucleotide (FAD) cofactor that participates in the catalytic activity in each subunit. To bind its steroid substrate, the enzyme uses lipid bilayers. In Fig. 1. The X-ray structure of the *Brevibacterium sterolicum* enzyme revealed two loops, 78-87 and 433-436, that function as a cover

for the active site and aid in substrate binding (**Akanksha *et al*., 2020).** It was thought that these loops would stretch, forming a hydrophobic channel between the membrane and the protein's active site and thereby removing the cholesterol substrate from the aqueous environment.t **(Vrielink and Ghisla., 2009).**

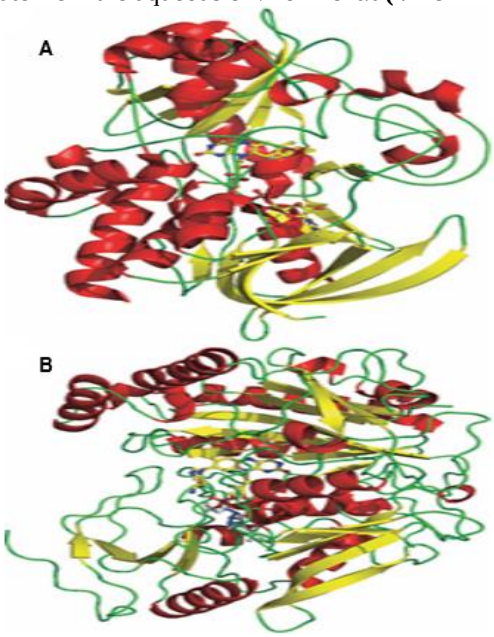


Fig. 1. Cholesterol oxidase 3D structure. (A) S Cholesterol Oxidase and (B) Bs Cholesterol Oxidase secondary structural elements appear in an overall view. The FAD cofactor is illustrated using a ball-and-stick graphic. **(Vrielink and Ghisla., 2009 and Akanksha *et al*., 2020).**

The identity of the catalytic residues and the evolutionary distribution of cholesterol oxidase are elucidated in Fig. (2). The enzyme can be found in two versions in various bacterial species: One with the FAD cofactor attached noncovalently to the enzyme and one with the cofactor bound covalently to the protein. image Compare and contrast the major biochemical properties of the two enzyme types. **(Vrielink and Ghisla., 2009)**

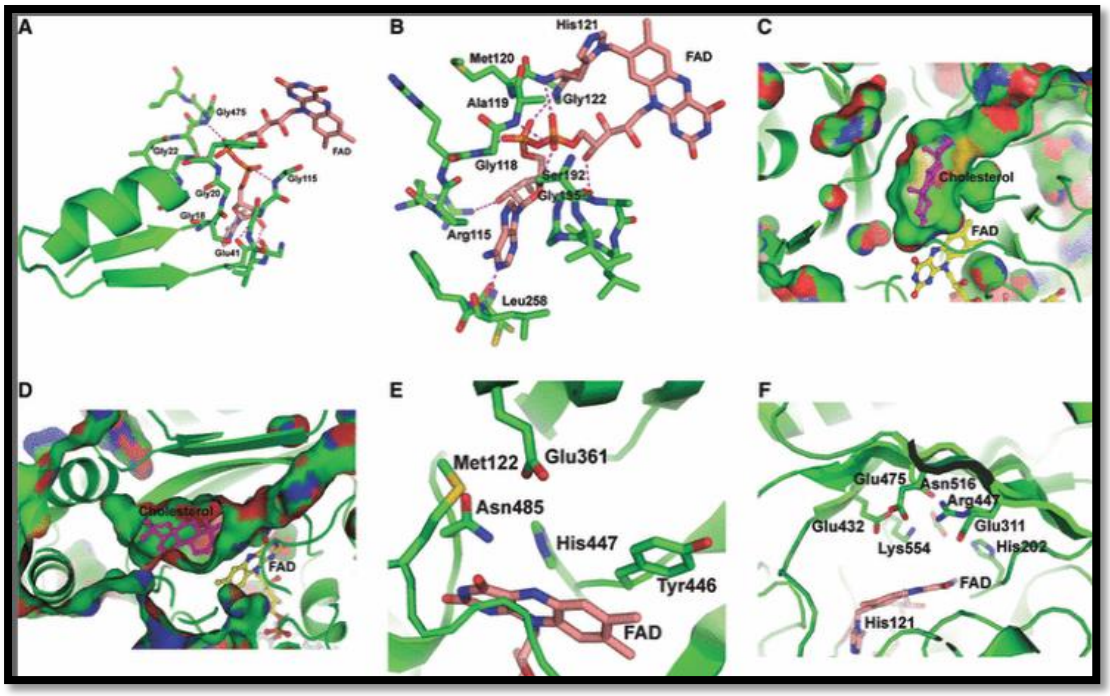


Fig 2. The structural viewpoints of various ChOx forms. (A) The protein and the FAD cofactor interact with the noncovalently linked enzyme ChOx (SChOx). (B) the covalently linked enzyme (BsChOx). Dashed lines represent the hydrogen bonds that form between the cofactor and the protein. Models of bound steroid in (C)

SChOx and (D) BsChOx active sites. (E) SChOx and (F) BsChOx active site residues **(Vrielink and Ghisla., 2009).**

8- Opportunities for increasing the stability of the cholesterol oxidase enzyme:

Adsorption/carrier-binding, covalent binding, polymer entrapment, and cross-linking are only a few of the ways utilised to immobilize enzymes. **(Bayraktar & Serilmez, 2011)**. The immobilization of cholesterol oxidase on nanomaterials is discussed, as well as other aspects. It has been demonstrated that immobilizing enzymes on Nano matrices improves their stability and activity. Because of their enormous surface area and compact size, Nanoparticles have been used as an immobilization matrix for enzymes. The biocompatible length scales and surface chemistry of nanoparticles allow for reusability, stability, and increased performance of enzyme-nanoconjugates. **(Ghosh *et al.*, 2018).**

Perdani et al. (2020) discovered that employing an immobilized enzyme to oxidize cholesterol indicated how the addition of a support material can substantially influence the enzyme's ability to oxidize the substrate. Text changes are marked in orange, and you can add more by clicking on individual words and replacing them with synonyms. The surface and mesoporous cages of the MOF PCN-333(Al), used as a colorimetric biosensor for cholesterol detection, were studied. were co-immobilized with cholesterol oxidase and HRP. **(Zhao *et al.*, 2019).** Tris (2,2-bipyridyl) dichloro-ruthenium (II) hexahydrate (Ru (bpy) 3Cl2) was found in Fe3O4&SiO2-SiO2-COD nanoparticles and might be used as a sensing material in multi-parameter fibre optic biosensors based on enzyme catalysis and oxygen consumption. (**Huang *et al.*, 2015). Hwang and Lee (2019)** discovered three key ways for immobilizing numerous enzymes: random co-immobilization, positional co-immobilization, and compartmentalization. Furthermore, graphene oxide hybrids can be used to produce (reduced) multi-enzyme systems for sensitive biosensing (**Xu *et al.*, 2020**). Biosensors are tools that translate biological systems' physical, biological, and chemical signals into electrical signals by pinpointing precise reactions to specific analytes. Cholesterol esterase and cholesterol oxidase are two enzymes that are employed. Biosensor surfaces have been modified to increase sensitivity to biological moieties in order to detect cholesterol levels in blood or human serums. Chitosan nanocomposites-based biosensors have been proven to be more effective, sensitive, and long-lasting than pure chitosan **(Murugesan and Scheibel, 2021**).

9- Molecular characterization of cholesterol oxidase from endophytic bacteria

In living organisms, many genes code for the manufacture of the cholesterol oxidase enzyme. The COX gene on chromosome 2 encodes the enzyme in humans.

The genes that encode for the generation of cholesterol oxidase in bacteria differ depending on the kind of bacteria. Molecular research approaches can be utilized to identify the genes responsible for enzyme production in many living species. PCR can be used to amplify certain DNA sections from the organism's genome that have been linked to the cholesterol oxidase gene. This can be accomplished by utilizing primers built based on the known sequence of the gene in other organisms. **Kanaparthi and Kandimalla (2018).**

Another technique that can be used to identify the cholesterol oxidase gene is to perform a homology search using informatics tools. This requires comparing the organism's genome to known cholesterol oxidase genes in other animals using tools such as BLAST (Basic Local Alignment Search Tool). (**Liu *et al.*, 2012; Yang *et al.*, (2018).**

The molecular characterization of cholesterol oxidase from endophytic bacteria involves the identification and isolation of the gene coding for the enzyme, followed by the characterization of the protein product **(Bharti & Ohri, 2018).** The first step in the molecular characterization of cholesterol oxidase from endophytic bacteria is the identification of the gene coding for the enzyme. This can be done by using PCR (polymerase chain reaction) to amplify a fragment of DNA that is specific to cholesterol oxidase. The amplified DNA can then be sequenced to identify the gene **(Sankari & Manivasagan, 2019).** After identifying the gene responsible for encoding the protein product, the next step is to isolate the protein. To do this, the gene can be produced in an appropriate host system, such as E. coli, and the protein extracted using chromatography techniques, as **Sharma et al. (2001)** explain. Once the protein has been purified, it can be characterized using various techniques such as SDS-PAGE, Western blotting, and enzyme activity assays. SDS-PAGE is a technique that separates proteins based on their size. When the purified cholesterol oxidase protein is run on a gel alongside molecular weight markers, its size can be estimated, as described by **Bharti and Ohri (2018)**. It is a technique that uses antibodies to detect specific proteins. The purified cholesterol oxidase protein can be transferred to a membrane and probed with an antibody specific to cholesterol oxidase to confirm its identity, as explained by **Sharma *et al.* (2001)**. Cholesterol oxidase activity may be determined using enzyme activity assays, which monitor the production of cholest-4-en-3-one from cholesterol in the presence of pure cholesterol oxidase protein. cholesterol oxidase activity can be assessed under a variety of circumstances, including temperature, pH, and substrate concentration, as detailed by **Sankari and Manivasagan (2019).**

Beside these techniques, other methods such as mass spectrometry can be used to analyze the purified protein to identify its amino acid sequence, post-translational modifications, and other structural and functional properties, as described by **Bharti and Ohri (2018).** Overall, the molecular characterization of cholesterol oxidase

from endophytic bacteria involves identifying and isolating the gene encoding the enzyme, purifying and characterizing the protein product using various techniques, as explained by **Wang and Li (2011).** This information can be useful in understanding the function of cholesterol oxidase in endophytic bacteria and its potential applications in biotechnology, as discussed by **Zhang and Zhang (2014).** Cell-associated cholesterol oxidase overproduction and molecular characterization **Yamada *et al.* (2019)** isolated cholesterol oxidase from Streptomyces *lavendulae YAKB-15*.

10- Biotechnological applications cholesterol oxidases from endophytic bacteria

Cholesterol oxidase is an enzyme that catalyses the conversion of cholesterol to cholest-4-en-3-one, which has several biotechnological uses. Immobilising cholesterol oxidase can improve its stability, reusability, and activity, making it more efficient for industrial applications. These characteristics make cholesterol oxidase an excellent enzyme to use in industrial and clinical laboratory applications. Cholesterol oxidase has been extensively researched and commercialised for assessing cholesterol levels In clinical samples, bioconversion of cholesterol into useful compounds, food preparation, and insecticidal effect against cotton weevils. Cholesterol oxidase has been intensively explored and commercialised for measuring cholesterol levels in clinical samples, bioconversion of cholesterol into useful chemicals, food preparation, and insecticidal action against cotton weevils. **(Doukyu and Aono *et al.*, 2001; El-Naggar and El-Shweihy 2020).**

There are some medical uses of cholesterol oxidase produced by endophytic bacteria. Endophytic bacteria-produced cholesterol oxidase has been proven to effectively decrease cholesterol levels in animal models This is accomplished by converting cholesterol to cholest-4-en-3-one. which can be excreted from the body. This can potentially be used as a cholesterol-lowering therapy in humans. (**Shah and Khullar, 2017; Singh *et al.*, 2018**).

Because of its role in carcinogenesis, cholesterol has attracted more attention. Changes in cholesterol metabolism are implicated in cancer development, according to clinical and experimental findings **(Silvente and Poirot, 2012).** Cancer is often distinguished by aberrant and uncontrolled cell growth, apoptosis resistance, cell migration, and other essential traits. Furthermore, One of the new metabolic indicators is disturbance of lipid metabolism **(Liu *et al.*, 2017b).** 4-cholesten-3-one reduces breast cancer cell viability and changes EGFR expression in membrane rafts by inhibiting lipogenesis and boosting LXR-dependent cholesterol transporters. (**Elia *et al.*, 2019**). Statins' therapeutic effects in numerous malignancies, including prostatic, gastric, esophageal, and hepatic cancer, have been seen in current anticancer therapy techniques targeting cholesterol metabolism **Stryjkowska *et al.* (2015). El-Naggar *et al.* (2016)** demonstrate that cholesterol oxidase outperforms the currently used anticancer medication doxorubicin and that cholesterol oxidase may be utilised as a possible natural anticancer agent**.** An in vivo study found that the combined treatment reduced tumour development substantially more effectively than either the cholesterol oxidase or Dox treated groups alone. Furthermore, cholesterol oxidase treatment resulted in phosphorylation of JNK (c-Jun NH2-terminal kinase) and p38, downregulation of Bcl-2 (B-cell lymphoma/leukemia-2) and upregulation of Bax with the release of activated caspase-3 and cytochrome C, most likely due to the production of hydrogen peroxide along with cholesterol oxidation.. **Devi and Kanwar (2017).** Cholesterol, according to **Xiao *et al.* (2019)**, plays a crucial function in cancer formation. Clinical and experimental studies have shown that hypercholesterolemia and a high-fat, high-cholesterol diet can alter cancer development geranylation (GG), a critical branch of the cholesterol manufacturing route for the maintenance of breast cancer stem cells (CSC).

(extracellular signal-regulated kinase 1/2) which was irreversible
even a□er cholesterol addi□on. Further studies indicated that
Choletserol oxidase treatment also promoted the genera□on of reac□ve
oxygen species (These findings suggested that Choletserol oxidase leads to irreversible cell apoptosis by decreasing cholesterol content and increasing ROS level. This indicates that he microbial Choletserol oxidase may be a promising candidate for a novel anti-tumor therapy **[Liu et al 2014 ; Devi and Kanwar (2017)].**

Liu J, Xian G, Li M, Zhang Y, Yang M, et al (2014) Cholesterol oxidase
from Bordetella sp. promotes irreversible cell apoptosis in lung
adenocarcinoma by cholesterol oxida□on. Cell Death and Disease
5: 1372

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HOx treatment
inhibited phosphoryla□on of Akt (protein kinase B) and ERK1/2
(extracellular signal-regulated kinase 1/2) which was irreversible
even a□er cholesterol addi□on. Further studies indicated that
Choletserol oxidase treatment also promoted the genera□on of reac□ve

oxygen species (ROS). Cholesterol oxidase is used in the production of steroid hormones such as cortisol, aldosterone, and testosterone. It is used to convert cholesterol to cholest-4-en-3-one, which is a precursor for the synthesis of these hormones. It participates in bile acid biosynthesis. Cholesterol oxidase is used in the production of precursors of hormonal steroids from cholesterol as one of the physiological functions of this enzyme **(Kreit and Sampson, 2009; Hamed *et al.*, 2010)** For instance, it can be used for production of diagnostic kits to detect blood cholesterol, biological insecticide and precursors for steroid hormones (**Kim and Kim 2017; Puri and Kaur 2018**).

The enzyme has numerous uses, including biosensor production, steroid biotransformation and insect biocontrol, the manufacture of polyene macrolide pimaricin, and the giving of virulence to *Rhodococcus equi*, *Mycobacterium TB,* and *Mycobacterium leprae* (**kanwar and devi 2017**). A nanotechnology-based method could be effective in improving the clinical performance of cholesterol oxidase-based biosensors (**Narwal *et al.*, 2019 and Akanksha *et al*. 2020**).

Antibacterial therapy: Cholesterol oxidase produced by endophytic bacteria has been shown to have antibacterial properties. It works by disrupting the bacterial cell membrane, leading to bacterial cell death. **(Vinothkumar and Annamalai, 2018). Kharitonov and Shumyantseva (2019**) Reported that Cholesterol oxidase-based biosensors can be used in cancer diagnosis and therapy. Because cholesterol oxidase is widely utilised in the enzymatic assessment of total and free cholesterol in clinical samples, blood, and food, it has a high economic value. (**Hamed *et al.*, 2010; Molaei *et al.*, 2014**). **Vrielnik (2010)** It was revealed that the enzyme plays an important function in bacterial pathogenesis. as stated by **Navas *et al*. (2001),** *R. equi*, a horse pathogen that causes opportunistic human infections**,** produces a substantial membrane-damaging component called cholesterol oxidase. Furthermore, dangerous bacteria such as *Bordetella* have been discovered. (**Lin *et al.*, 2010**) and fast-growing *Mycobacteria* (**Yao et al., 2013)** require cholesterol oxidase to penetrate host cells, most likely because cholesterol oxidase has the ability to) change the physical structure of the cell membrane by converting cholesterol to cholesten-4-en-3-one. These bacteria- and fungus-specific enzymes might be the focus of a new class of antibiotics. Cholesterol oxidase has also been related to the start of several viral (HIV) and nonviral prion disorders (Alzheimer's). **Kumari and Kanwar (2012).** There is no mammalian equivalent of cholesterol oxidase. In Alzheimer's disease, however, beta-amyloids oxidise cholesterol at many carbon groups, including the C (3)-OH group, and catalytically create 4-cholesten-3-one, simulating cholesterol oxidase function (**Gamba *et al.*, 2011).**

Furthermore, The enzyme has been demonstrated to be involved in sterol and nonsteroidal chemical transformation (**Ahire *et al.*, 2012),** Membrane structure research, steroid hormone precursor synthesis, and the production of the polyene macrolide pimaricin (**Doukyu, 2008; Mendes et al., 2007).** The enzyme is also employed in the microanalysis of steroids in food samples to distinguish the steric configuration of 3-ketosteroids from 3-hydroxysteroids (**Kuppusamy and Kumar**, **2016**).

The immobilization of cholesterol oxidase on chitin, a natural biopolymer, was described in a paper published by **(Rastegari *et al.*, 2001**). The enzyme was immobilized via adsorption on the chitin surface, followed by glutaraldehyde cross-linking. The immobilized enzyme outperformed the free enzyme in terms of thermal stability and reusability, keeping more than 90% of its original activity after 10 reuse cycles.

Puri *et al*. (2018) present Another work that was presented was the immobilisation of cholesterol oxidase on magnetic nanoparticles. another study found that cholesterol oxidase could be immobilised on magnetic nanoparticles. The immobilization achieved by covalent bonding of the enzyme to the surface of the nanoparticles using a bifunctional linked molecule. The enzyme reacted with chitosan-aminated magnetite nanoparticles. The immobilized enzyme demonstrated greater temperature and pH stability, as well as the ability to preserve more than 80% of its initial activity after 10 cycles of reuse. The magnetic nanoparticles also made it possible to easily separate the enzyme from the reaction mixture, which is useful in industrial applications. At different reaction times, cholesterol oxidase immobilised with chitosan magnetite was able to oxidise up to 90% of a cholesterol substrate. The NH2 functional group was identified during the immobilisation step. (**Senthilkumar & Gomathi 2018).** Polymers including PEG, painted polystyrene (PS) nanoparticles, and Congo red have also been employed to immobilise cholesterol oxidase. (**Ghosh *et al.*, 2018**). Cholesterol Oxidase was immobilised in Chitosan Magnetite Material for Biosensor Application by **Perdani et al., (2020).**

Conclusion

Because cholesterol oxidase is widely used, the search for low-cost culture conditions for overproduction of this enzyme is continuing. Among the many microbes are fungus and bacteria appear to be the most capable of producing enzymes in solid substrates. we think that dextran and sodium alginate can be used as immobilizing agent for conjugation with cholesterol oxidase from endophytic bacteria for improving its enzymatic properties, reusability and operational stability for using in industrial applications, Agricultural as insect pests and medical application as diagnosis agent. The major purpose of this work is to present an overview of the current research state of cholesterol oxidase generated by endophytic bacteria. while also identifying potential future research possibilities. Through this review, readers can obtain a deeper understanding of the progress made so far in this field, as well as the potential opportunities for further research.

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