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Enhancement of Cholesterol Oxide Production by *Rhodococcus UCC0018* via Optimization of Fermentation Conditions

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ABSTRACT

Cholesterol oxidase is an important enzyme that catalyzes the oxidation of cholesterol to cholest-4-en-3-one, with the simultaneous reduction of oxygen to hydrogen peroxide. It has gained significant attention due to its wide range of applications in medical diagnostics, pharmaceuticals, and food processing. Cholesterol oxidase is crucial in reducing cholesterol levels by breaking it into less harmful byproducts, making it a valuable tool in managing cholesterol-related health issues. The enzyme is primarily produced through microbial fermentation using microorganisms. However, the yield and efficiency of cholesterol oxidase production are highly dependent on fermentation conditions, including temperature, pH, and agitation rate, which significantly influence microbial growth, enzyme stability, and overall productivity. The initial production of cholesterol oxidase and cell growth under non-optimized conditions revealed suboptimal performance, with enzyme activity and cell dry weight (DCW) peaking at lower levels. To address this, optimization of fermentation conditions was conducted in a 250 ml flask. The evaluated temperature range was 30°C to 35°C, the pH was optimized to a range of 7.5 to 8.0, and agitation was optimized at 150 to 200 rpm. Under these optimized conditions (35°C, 7.5 pH, 150 rpm), cholesterol oxidase activity reached a peak of 3.5 U/mL, a substantial increase compared to pre-optimization levels. Cell growth also showed marked improvement (1.936 g/L), indicating robust microbial activity. The findings provide valuable insights into the fermentation process, emphasizing the importance of parameter control in industrial enzyme production. The optimized conditions not only enhanced enzyme yield but also improved the overall efficiency of the bioprocess, offering a scalable approach for the industrial production of cholesterol oxidase. This enzyme has significant potential in addressing high cholesterol levels in the bloodstream, contributing to better management of cardiovascular health.

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1. Introduction

Cholesterol oxidase (CHO) is a key enzyme with wide applications in the pharmaceutical, food, and diagnostic industries, particularly in cholesterol metabolism and biosensor development [1,2]. This enzyme catalyzes the oxidation of cholesterol to cholest-4-en-3-one, with simultaneous hydrogen peroxide production, making it valuable for cholesterol quantification in clinical diagnostics [3]. The growing concern over hypercholesterolemia and cardiovascular diseases has further amplified interest in microbial cholesterol oxidase as a potential biocatalyst for cholesterol degradation and medical applications [2].

Microbial fermentation is a widely adopted method for cholesterol oxidase production due to its cost-effectiveness and scalability. Various bacterial species, including *Rhodococcus, Streptomyces*, and *Brevibacterium*, have been identified as potent producers of this enzyme [3,4]. Among these, *Rhodococcus* strains exhibit significant advantages due to their metabolic flexibility, robustness, and high enzyme yield [5]. However, optimizing fermentation conditions remains crucial to achieving maximum enzyme production. Factors such as temperature, pH, medium component, aeration, and agitation influence microbial growth, enzyme secretion, and overall productivity [6-8].

Several studies have reported that optimizing environmental and nutritional conditions can enhance cholesterol oxidase production. For instance, adjusting the fermentation temperature has been shown to stabilize enzyme structure and prevent thermal denaturation, thus improving enzyme activity [9,10]. Similarly, optimizing pH can enhance microbial metabolism, as cholesterol oxidase activity is often sensitive to pH fluctuations [11]. Furthermore, aeration and agitation play vital roles in oxygen transfer, which is essential for microbial respiration and enzyme biosynthesis. Similarly, cholesterol oxidase production by *Streptomyces anulatus* was enhanced through optimization of agitation speed and medium composition [12]. Despite these advancements, further research is needed to develop a systematic approach for optimizing cholesterol oxidase production, ensuring its viability for large-scale applications.

This study focuses on optimizing the fermentation parameters for *Rhodococcus* UCC0018 to enhance cholesterol oxidase production. Specifically, the research examines the effects of temperature, pH, and agitation on enzyme activity and microbial growth. By refining these parameters, this study aims to improve enzyme yield while maintaining industrial feasibility. The findings will contribute to the development of scalable and cost-effective fermentation strategies, advancing the commercial production of cholesterol oxidase for biomedical and industrial applications. The optimized fermentation conditions enhance cholesterol oxidase (CHO) production, making it more viable for industrial applications. In biotechnology, this improves enzyme yield and reduces production costs, enabling large-scale bioreactor implementation. In pharmaceuticals, higher CHO availability supports cholesterol biosensors and potential drug formulations. The food industry benefits from CHO's use in low-cholesterol food processing, meeting consumer health demands. Additionally, sustainable bioprocessing reduces resource consumption and waste. These findings bridge laboratory research and industrial application, supporting cost-effective, high-yield CHO production with potential for genetic and bioreactor-based enhancements in future studies.

Regardless of the advancements in microbial cholesterol oxidase production, challenges remain in optimizing fermentation conditions for maximum yield and industrial scalability. Previous studies have focused on individual factors such as temperature, pH, and agitation, but a comprehensive optimization strategy integrating these parameters remains limited. Additionally, while *Rhodococcus* species are known for their metabolic versatility, their potential for large-scale cholesterol oxidase production under optimized conditions has not been fully explored. This study addresses these gaps by systematically optimizing key fermentation parameters (temperature, pH, and agitation) to

enhance cholesterol oxidase production in *Rhodococcus UCC0018*. By bridging this knowledge gap, the research aims to provide a scalable and cost-effective approach for industrial enzyme production, contributing to advancements in biotechnology and pharmaceutical applications.

2. Methodology

2.1. Microorganism and Inoculum Preparation

The bacterium, *Rhodococcus* UCC 0018 was used throughout this study. This bacterium was isolated from a petroleum contaminated soil at Port Dickson, Negeri Sembilan, Malaysia and maintained at the UNISEL Culture Collection Centre, Selangor, Malaysia. The bacterium from the stock culture was grown in nutrient broth for 24 h and the culture was used as standard inoculums for all cultivations. Culture was stored in -80 ^{o}C for subsequent used.

2.2. Medium

Cholesterol salt medium (CSM) consisted of (g/L): NH4NO3, 17; K2HPO4, 0.25; MgSO4.H2O, 0.25; FeSO4.H2O, 0.001; NaCl, 0.05; cholesterol, 10; agar 20 and Tween 80, 0.01% (v/v) was used as the basal medium for the cultivation of *Rhodococcus* UCC0018. The pH was adjusted to 7.0. Dry autoclaved technique was used to avoid cholesterol coagulation. Cholesterol powder was autoclaved separately in 25ml Schott bottle and let to cool in room temperature for 10 minutes before adding it with the autoclaved MSC media in sterilised environment.

The cultivation of *Rhodococcus* UCC0018 was carried out in 250 mL shake flask containing 100 mL medium. The flask was inoculated with 10% (v/v) inoculum and incubated at 30 ^{o}C on a rotary shaker (Jeiotech; SI-600R, Korea), agitated at 160 rpm for 24 h. At the end of cultivation, the cells from the culture broth were harvested by centrifugation at 29,568 g (TOMY; MX-305, Japan) for 15 min. Supernatant was discarded and the cells.

2.3. Fermentation

Batch fermentation in was carried out in 250 mL shake flask containing 100 mL medium. Medium was sterilised at 121^{o}C for 20 minutes. The medium was inoculated with 10% (v/v) inoculum that was previously grown overnight on the nutrient broth medium (NB) to initiate the cultivation and production of cholesterol oxidase. The flask was incubated at temperature 30^{o}C on a rotary shaker, agitated at 160 rpm. All fermentations were performed in triplicate and the results were presented in average value. During the cultivation, 10 mL of culture samples were withdrawn at different time intervals for analysis of cell concentration and cholesterol oxidase

2.4. Analytical Methods

The optical density of the culture was measured at 680 nm using a spectrophotometer. Cell concentration in term of dry cell weight was determined by filtration and oven dried method. The known volume of culture sample was filtered through a known weight of dried membrane filter with the pore size of 0.25 μm using vacuum pump. The membrane filters with the bacterial cells were at 80^{o}C for at least 24 h, until a constant weight was achieved.

2.5 Quantitative Analysis of CHO

Enzyme activity of the crude CHO and purified CHO extracted from isolates were tested spectrophotometrically using peroxide and o-dianisidine method. Hydrogen peroxide generated during cholesterol oxidation process was measured in this method. In this reaction, hydrogen peroxide was coupled with o-dianisidine in presence of enzyme peroxidase, which causes oxidation of o-dianisidine. Oxidized odianisidine has maximum absorption at 500nm. Increase in the absorbance for reaction mixtures was recorded and calculations for enzyme units were done.

(Sigma s Quality Control Procedure, 1994). For enzymatic assay of CHO produced by isolated organisms, following reagents were used.

i. Reagent A (Buffer): 50mM Potassium Phosphate Buffer, pH 7.5 at 25°C (Prepared 100 ml in distilled water using KH2PO4. Adjusted to pH 7.5 with 1 M KOH)
ii. Reagent B (ODA): 1% (w/v) o-Dianisidine Solution (Prepared freshly in 5 ml distilled water using of DianisidineDihydrochloride, Himedia)
iii. Reagent C (Chol): 0.5% (w/v) Cholesterol- Hi-media with 10% (v/v) Triton X-100 Solution (Prepared by initially dissolving the Cholesterol in 10 ml of Triton X-100.Heated until the solution clarifies. Then 90 ml of distilled water was added. Vortexed and stored the solution at 4°C)
iv. Reagent D (POD): Peroxidase Enzyme Solution (A solution was prepared immediately before use, using Horseradish Peroxidase, Chromus Biotech Ltd. containing 100 units/ml in distilled water i.e. 0.1mg in 3.3ml)
v. Reagent E (Enzyme Solution i.e. supernatant of broth): Cholesterol Oxidase Enzyme Solution (Here, we used the supernatant that was obtained by centrifugation of the culture broth at 10000×g for 10 min at 4°C).

A reaction cocktail was prepared by pipetting (in milliliters) the following reagents into a conical flask: Reagent A (Buffer) 40.0ml, Reagent B (ODA) 0.50ml. These reagents were mixed thoroughly by swirling and pH was adjusted to 7.5 at 25°C with 100mMHCl or 100 mM KOH. Then Reagent A was added to make a final volume of 50 ml. The contents were mixed by swirling thoroughly and oxygenated for 10 minutes immediately before use. Further the quantitative estimation of cholesterol was performed as given below:
The increase in absorbance was recorded every minute for 5 min and the following formula was used to calculate the enzyme activity:

$$\frac{\text{Units}}{\text{ml}}\text{enzyme} = \frac{\left(\frac{\Delta A_{500\text{nm}}}{\min \text{Test}} - \frac{\Delta A_{500\text{nm}}}{\min \text{Blank}}\right)}{(7.5)(0.1)} \times (3)(\text{df}) \quad (1)$$

where, 3 = the total volume of the assay, df = dilution factor, 7.5 = milimolar extinction coefficient of o-Dianisidine, 0.1 = volume (in ml) of enzyme used.

3. Results

3.1 Effect of Temperature

The study evaluated temperature ranges from 30°C to 35°C, with the optimal condition determined at 30°C was shown in Figure 1. The effect of different incubation temperatures (20°C, 25°C, 30°C, 35°C, 40°C and 45°C) on cholesterol oxidase (CHO) production and microbial growth rate in *Rhodococcus UCC0018* was evaluated. The results indicate that temperature plays a crucial role in both enzyme activity and microbial proliferation. Among the tested conditions, 35°C was the optimal temperature, yielding the highest CHO activity (0.521 U/mL) and microbial biomass (0.775 g/L), indicating maximum metabolic efficiency. The correlation between enzyme production and microbial growth suggests that optimal temperature 35°C enhances both cell viability and enzymatic activity, as observed in previous studies on *Rhodococcus erythropolis* PR4 [13]. Meanwhile *Bacillus pumilus* W8 and *Castellaniella* sp showed optimum temperature at 35°C and 40°C, respectively [14,15].

At lower temperatures [20°C and 25°C], microbial growth rates were reduced, leading to lower CHO production. At 20°C, enzyme activity was significantly suppressed, likely due to slower metabolic processes and limited nutrient uptake, which hindered biomass accumulation. Although 25°C showed a slight improvement in CHO activity compared to 20°C, it was still lower than at 30°C, confirming that suboptimal temperatures delay microbial replication and enzyme biosynthesis. Reduced microbial growth at these temperatures directly affects cholesterol oxidase yield, as enzyme production is closely linked to active biomass.

Additionally, at 40°C, microbial growth declined sharply, leading to a significant reduction in CHO activity. The lower biomass observed at this temperature suggests that elevated heat stress impairs cell viability, reducing enzyme synthesis. High temperatures likely caused protein misfolding and enzyme denaturation, leading to structural instability and reduced catalytic efficiency. Additionally, heat-induced stress responses in *Rhodococcus UCC0018* may have shifted metabolic pathways toward survival mechanisms rather than enzyme production [16]. The decline in microbial growth rate at 40°C further confirms that excessive heat negatively impacts both cellular division and metabolic performance.

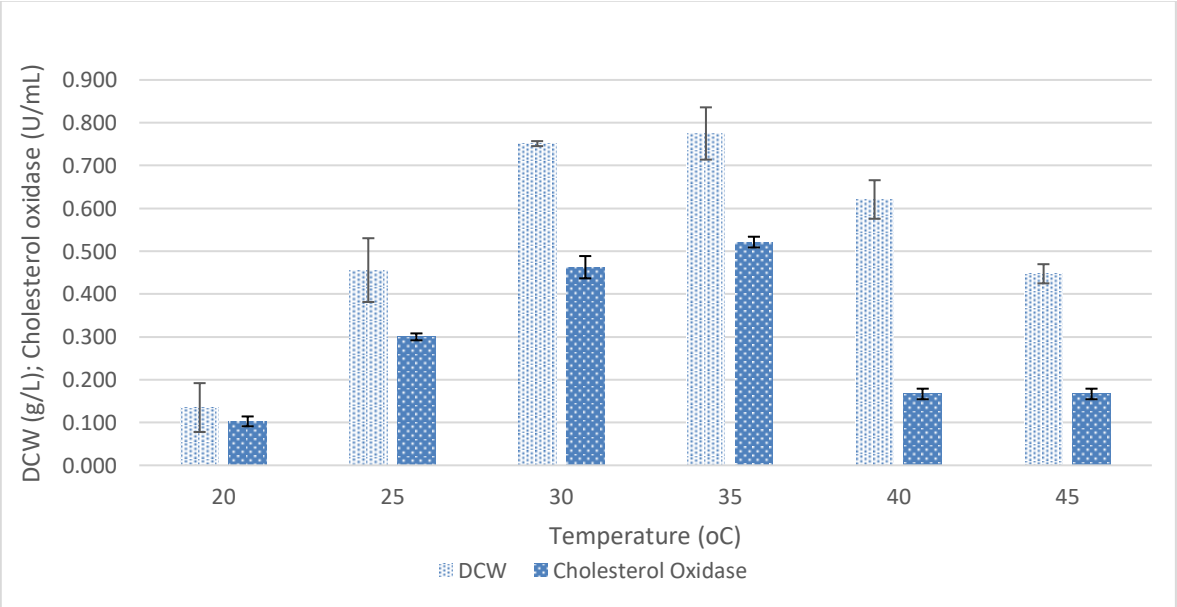

Fig. 1 Effect of incubation temperature on cholesterol oxidase production and *Rhodococcus UCC0018* growth. Error bars represent standard deviations (n=3)

3.2 Effect of pH

The effect of pH variations (6.0,7.0, 7.5, 8.0, 9.0 and 9.5) on enzyme activity was examined as shown in Figure 2, with optimal activity observed at pH 7.5. pH is a crucial factor in microbial fermentation as it influences enzyme stability, metabolic activity, and nutrient availability. The results indicate that pH 7.5 was the optimal condition, yielding the highest CHO activity (0.783 U/mL) and microbial biomass (0.641 g/L). This suggests that *Rhodococcus UCC0018* exhibits maximum enzymatic efficiency under slightly alkaline conditions, which aligns with previous studies on *Rhodococcus* species, where neutral to mildly alkaline pH ranges were found to support optimal enzyme production.

At pH 6.5 and 7.0, cholesterol oxidase production was significantly lower. The reduced enzyme activity at pH 6.5 suggests that acidic conditions negatively impact microbial metabolism, likely disrupting protein folding and enzyme stability. Additionally, microbial growth rates were lower in these conditions, indicating that an acidic environment affects cell viability and replication. While pH 7.0 showed an improvement in enzyme production compared to pH 6.5, it remained lower than the peak activity observed at pH 7.5. This trend supports findings that most *Rhodococcus* strains favor neutral to slightly alkaline conditions for optimal growth and enzyme biosynthesis [16].

Optimization of culture conditions for *Streptomyces olivaceus* MTCC 6820 increased CHO production from 1.9 U/mL to 4.2 U/mL, with optimal pH at 7.5 [8]. For *Pseudoartrobacter scleromae*, maximum CHO production (0.088 U/mL) was observed at pH 8.0, 28°C, and 72 hours of cultivation [17]. Another study on Malaysian *Rhodococcus* isolates found that strain UCC0021 produced the highest CHO activity of 1.180 U/mL after 72 hours, which was 48% higher than other actinobacteria [16]. These findings highlight the potential of various bacterial strains for CHO production under optimized conditions.

While, at pH 8.0, a decline in CHO activity and microbial biomass was observed. Although *Rhodococcus* UCC0018 can tolerate mildly alkaline conditions, excessive alkalinity may disrupt cellular homeostasis, leading to enzyme instability and metabolic stress. The decrease in microbial growth at pH 8.0 further supports this, as elevated pH levels may reduce nutrient solubility and interfere with enzyme-substrate interactions, ultimately hindering cholesterol oxidase synthesis.

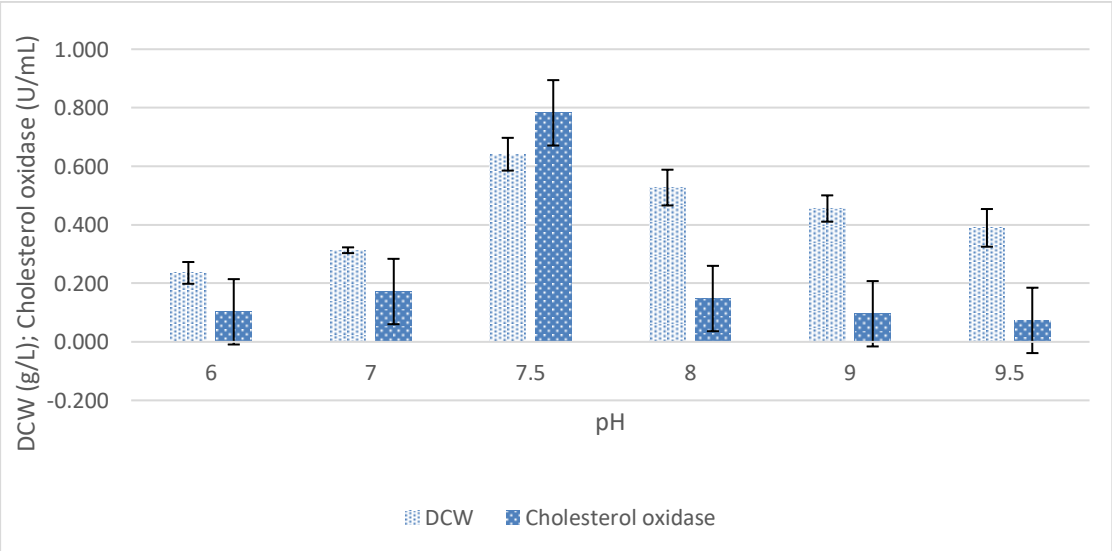

Fig. 2 Effect pH on cholesterol oxidase production and *Rhodococcus UCC0018* growth. Error bars represent standard deviations (n=3)

3.3 Effect of Agitation

The effect of different agitation speeds (100 rpm, 150 rpm, 200 rpm, and 250 rpm) on cholesterol oxidase (CHO) production and microbial growth in *Rhodococcus UCC0018* was evaluated and shown in Figure 3. Agitation plays a critical role in microbial fermentation by influencing oxygen transfer, nutrient distribution, and the overall metabolic activity of microbial cells. The results indicate that 150 rpm was the optimal agitation speed, yielding the highest CHO activity (2.119 U/mL) and the greatest microbial biomass production (1.936 g/L). This suggests that at 150 rpm, oxygen availability was sufficient to support aerobic respiration and enzyme biosynthesis while minimizing shear stress on the microbial cells. Similar findings have been reported in previous studies where optimal agitation improved enzyme production by balancing oxygen transfer and mechanical stress [9, 18]. Gómez-Ríos *et al.,* [19] investigated the effects of shear stress on clavulanic acid production by *Streptomyces clavuligerus*, finding that high shear stress conditions enhanced oxygen availability and precursor synthesis. These studies highlight the critical role of agitation in balancing oxygen transfer and mechanical stress, with optimal speeds around 150 rpm supporting aerobic respiration and enzyme biosynthesis while minimizing cellular damage.

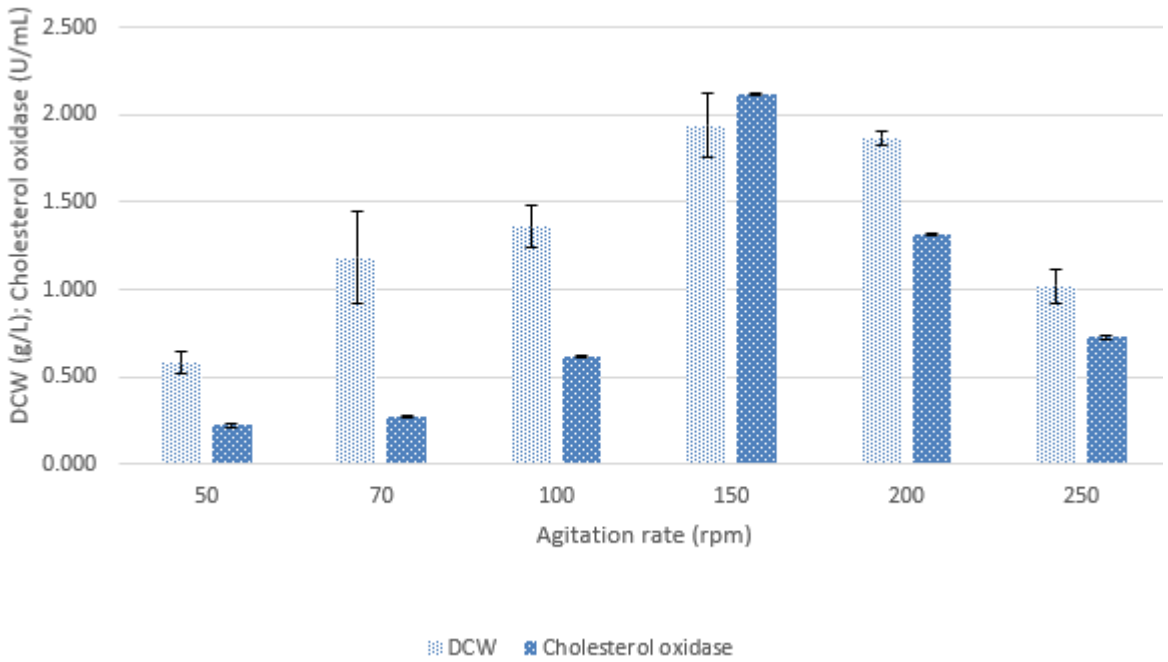

Fig. 3: Effect of agitation rate on cholesterol oxidase production and *Rhodococcus UCC0018* growth. Error bars represent standard deviations (n=3)

At 100 rpm, enzyme activity and microbial growth were significantly lower, indicating that insufficient agitation limited oxygen transfer. As *Rhodococcus UCC0018* is an aerobic microorganism, oxygen availability is crucial for its metabolism and cholesterol oxidase production. The reduced CHO activity at 100 rpm suggests that the lower aeration rate restricted microbial respiration, leading to suboptimal metabolic activity and enzyme biosynthesis. Additionally, nutrient distribution in the fermentation medium may have been uneven, further limiting microbial growth and CHO production. These observations are consistent with previous studies that have demonstrated the importance of adequate aeration in microbial fermentation for enzyme synthesis (20).

Meanwhile, at higher agitation speeds of 200 rpm and 250 rpm, a decline in cholesterol oxidase activity and microbial biomass was observed. Although increased agitation generally enhances oxygen transfer, excessive agitation can lead to mechanical shear stress, damaging microbial cells and reducing enzyme production efficiency. At 250 rpm, the significant drop-in enzyme activity suggests that the microbial cells experienced physical stress, which may have disrupted cell membranes and enzyme stability. Additionally, excessive agitation can lead to increased foaming and gas stripping, reducing the availability of dissolved oxygen despite higher mixing rates. However, optimal agitation speeds varied depending on the organism and process. For *Rhodococcus equi*, maximum cholesterol oxidase activity was achieved at 200-300 rpm [21].

The optimization of fermentation parameters significantly enhanced cholesterol oxidase (CHO) production in *Rhodococcus UCC0018* as shown in Table 1. Under non-optimized conditions, enzyme activity and microbial growth were suboptimal, necessitating parameter adjustments. After optimization, cholesterol oxidase activity reached 3.5 U/mL, a substantial increase from pre-optimization levels. This enhancement aligns with previous findings where optimized fermentation conditions led to improved enzyme production in *Rhodococcus* strains [8].

Table 1
Enhanced cholesterol oxidase production by *Rhodococcus UCC0018* under optimized conditions (35°C, pH 7.5, 150 rpm)

Hours	DCW (g/L)	CHO (U/mL)
0	0.379	0.17
12	0.793	0.20
24	1.457	1.28
36	1.584	1.65
48	1.758	1.92
60	**1.936**	2.34
72	1.793	**3.50**
84	1.756	3.09
96	1.699	3.04
108	1.724	2.99

Compared to previous studies on cholesterol oxidase production, this research demonstrated competitive yields under optimized conditions. The enzyme activity of 3.5 U/mL better than some earlier reports on other microbial strains cultivated under optimized conditions (e.g., 1.86 U/mL) [22]. The significant improvement in enzyme production suggests that fermentation control plays a critical role in enhancing microbial enzyme biosynthesis. Temperature also shows significant effect when studies for antioxidant properties [23].

The optimized conditions not only enhance cholesterol oxidase yield but also offer potential scalability for industrial applications. Cholesterol oxidase has crucial applications in biosensors for cholesterol detection and biocatalysis in pharmaceutical processes. The findings highlight the importance of fermentation control for maximizing enzyme production while maintaining cost efficiency. Additionally, integrating bioreactor-based optimizations with genetic modifications may further enhance CHO productivity.

This study successfully optimized fermentation parameters, leading to a significant improvement in cholesterol oxidase production. The results provide insights into microbial enzyme synthesis and pave the way for further scale-up studies. Future research should explore bioreactor-based optimizations and genetic modifications to further enhance CHO productivity.

4. Conclusions

This study successfully optimized fermentation parameters for enhancing cholesterol oxidase (CHO) production in Rhodococcus UCC0018. Through systematic adjustments of temperature, pH, and agitation, enzyme activity was significantly improved, reaching a peak of 3.5 U/mL under optimized conditions. The findings highlight the critical role of fermentation control in maximizing microbial enzyme yield, with optimal conditions enhancing both enzyme activity and cell growth.

The results demonstrated that maintaining temperature at 30°C, pH at 7.5, and agitation at 150 rpm created the most favorable conditions for microbial metabolism and enzyme biosynthesis. These findings are consistent with previous studies emphasizing the importance of precise environmental regulation in microbial fermentation. Furthermore, the study underscores the significance of oxygen transfer and nutrient availability in promoting cholesterol oxidase production, supporting its potential for industrial-scale applications.

The broader implications of this research extend to biomedical, pharmaceutical, and food industries, where cholesterol oxidase plays a vital role in cholesterol management, biosensor development, and biocatalysis. The optimized fermentation strategy not only enhances enzyme yield but also offers a scalable and cost-effective approach for future industrial applications. Future studies should explore bioreactor-based optimizations and genetic modifications to further enhance CHO productivity, ensuring its commercial viability in enzyme-based technologies.

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