Original Research Article

OPTIMIZATION, PURIFICATION AND CHOLINESTERASE ENZYME PRODUCTION: A BIOCHEMICAL AND STATISTICAL ANALYSIS

ABSTRACT

Cholinesterase enzymes play a vital role in regulating neurotransmission by hydrolyzing acetylcholine and other choline-based esters. This study focuses on the purification and optimization of cholinesterase enzyme production, integrating biochemical and statistical approaches to enhance yield and efficiency. The enzyme was first isolated from a selected microbial strain and subjected to a series of purification steps, including ammonium sulphate precipitation, dialysis and chromatographic techniques. The purified enzyme was then characterized for its specific activity, stability and kinetic parameters to evaluate its functional efficiency. To further enhance enzyme production, an optimization process was carried out using a statistical design approach. Response Surface Methodology (RSM) was employed to analyse key factors influencing enzyme yield, such as pH, temperature, incubation time, substrate concentration and agitation speed. A Central Composite Design (CCD) was applied to determine the optimal conditions for maximizing cholinesterase production. The optimized parameters resulted in a significant increase in enzymatic activity compared to the unoptimized process. Biochemical characterization revealed that the purified cholinesterase exhibited high thermal and pH stability, with an optimal activity range suited for industrial and medical applications. This study provides a comprehensive insight into the efficient purification and statistical optimization of cholinesterase production, paving the way for its potential applications in neurodegenerative disease research, pesticide detoxification, and biosensor development. The integration of

Commented [AA1]: why Related data Biochemical characterization revealing that the purified cholinesterase exhibits high thermal and pH stability, with an optimal activity range suitable for industrial and medical applications is not shown in the abstract

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biochemical and statistical methodologies ensures a robust and scalable approach for enhanced cholinesterase production.

Keywords: Cholinesterase Purification; Enzyme Optimization; Biochemical Characterization; Response Surface Methodology (RSM); Neurodegenerative Disease Research

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INTRODUCTION

Cholinesterase is essential enzyme responsible for the hydrolysis of acetylcholine and other choline esters, playing a crucial role in neurotransmission and neuroregulation. These enzymes are primarily categorized into Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE), they have significant physiological and pharmacological importance. AChE is predominantly found in the central and peripheral nervous systems, facilitating the rapid breakdown of acetylcholine to terminate nerve impulse transmission. BChE, on the other hand, is widely distributed in plasma, liver and other tissues, functioning as a detoxification enzyme by hydrolyzing various xenobiotic esters, including certain drugs and pesticides. Due to their vital roles, Cholinesterases are widely studied for their applications in neurodegenerative diseases, toxicology, drug development and biosensors. (Goldsmith *et al.*, 2017)

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The demand for high-purity cholinesterase enzymes has increased significantly, necessitating efficient optimization and purification strategies to enhance enzyme production. Traditional purification methods include ammonium sulphate precipitation, dialysis, and chromatographic techniques such as ion-exchange and affinity chromatography. These approaches help in obtaining cholinesterase with higher purity and specific activity. However, optimizing enzyme production requires precise control over various parameters such as pH, temperature, substrate concentration, incubation time, and agitation speed. The use of statistical models, particularly Response Surface Methodology (RSM) and Central Composite

Design (CCD), has proven effective in maximizing enzyme yield by systematically evaluating and fine-tuning these parameters. (Ashani *et al.,* 2016)

Biochemical characterization of the purified cholinesterase is critical for understanding its stability, kinetic behaviour and structural properties. Enzyme kinetics, including the determination of Michaelis-Menten constant (Km) and maximum velocity (Vmax), provide insights into substrate affinity and catalytic efficiency. Additionally, the thermal and pH stability of the enzyme influences its applicability in industrial and pharmaceutical fields. Cholinesterase inhibitors, widely used in Alzheimer's disease treatment and pesticide detection, further highlight the significance of purified cholinesterase in biomedical research. (Hiblot *et al.,* 2015)

This study aims to optimize and purify cholinesterase enzyme production using a combination of biochemical and statistical approaches. The integration of traditional purification techniques with modern statistical optimization ensures a cost-effective and scalable process for obtaining high-quality cholinesterase. The findings from this study will contribute in improving enzyme production efficiency, enabling its broader applications in Neuroscience, Biotechnology, and Environmental Toxicology. By refining purification and optimization strategies, this research enhances the feasibility of large-scale cholinesterase production, thereby expanding its industrial and clinical potential. (Goldenzweig *et al.,* 2016)

METHODOLOGY

Optimization of Culture Conditions

The optimization of culture conditions was carried out to maximize cholinesterase enzyme production. This involved identifying and refining key parameters influencing microbial growth and enzyme yield. A statistical approach was employed using Response Surface Methodology (RSM) to analyse the effects of temperature, pH, choline chloride

concentration, ammonium chloride concentration and incubation time on enzyme production. (Bigley *et al.*, 2015)

Selection of Microbial Isolates

A total of 125 microbial isolates were screened for cholinesterase (ChE) activity. Among them, sample No. 72 exhibited the highest enzyme activity and was selected for further optimization. The production media used for culture growth was Cheng's media, known for its efficacy in promoting enzyme synthesis. (Lockridge, 2015)

Statistical Optimization Using DoE

The production of cholinesterase enzymes requires precise optimization to enhance yield, stability and activity. Statistical methods such as Design of Experiments (DoE) provide a systematic and efficient approach in optimizing various physicochemical and biological parameters influencing enzyme production. DoE is a powerful statistical tool that evaluates multiple factors simultaneously, identifying their significance and interactions while minimizing the number of experimental runs. This method is particularly advantageous compared to traditional One-Factor-AT-a-time (OFAT) approaches, as it reduces experimental workload and provides more reliable optimization results. By implementing DoE, researchers can determine the optimal conditions for microbial growth, substrate utilization and enzyme secretion, ensuring a cost-effective and scalable production process.

Several key parameters are typically optimized using DoE in Cholinesterase enzyme production. These include the selection of appropriate carbon and nitrogen sources, which serve as essential nutrients for microbial metabolism and enzyme secretion. Additionally, pH and temperature play a crucial role in maintaining enzyme stability and activity, while incubation time is optimized to ensure peak enzyme production. Other factors such as agitation speed, aeration rate and inoculum size significantly influence enzyme yield by affecting oxygen transfer and microbial growth dynamics. Optimizing these parameters using

DoE leads to enhanced enzyme production, making the process more efficient and reproducible.

To achieve optimal enzyme yield, different experimental designs within DoE are employed. Plackett-Burman Design (PBD) is often used for screening to identify the most influential factors. Once key variables are determined, Response Surface Methodology (RSM) is applied to explore their interactions and optimize their levels. Among RSM designs, Central Composite Design (CCD) and Box-Behnken Design (BBD) are widely used for model development and optimization. These statistical approaches provide a predictive model that enhances the calibration of the conditions to maximize enzyme activity and stability.

The advantages of DoE in cholinesterase enzyme production are significant. It not only enhances enzyme yield but also reduces the cost and time associated with traditional optimization techniques. By systematically analyzing multiple parameters, DoE improves process reproducibility and scalability for industrial applications. Moreover, it enables a deeper understanding of factor interactions, which might not be evident through conventional methods. The integration of DoE with biochemical analysis provides a robust framework for advancing cholinesterase enzyme research, facilitating its applications in Pharmaceuticals, Biodegradation, and Neurobiological studies.

A Design of Experiment (DoE) approach was applied to evaluate the impact of multiple factors on enzyme production. The software predicted design (CCD) was used, with five independent variables, each assigned high (+1) and low (-1) levels:

Table 1: Statistical Optimization Using DoE

Variable	Low (-1)	High (+1)
Choline Chloride (%)	0.10	0.40
Ammonium Chloride (M)	0.01	0.05
pH	7.0	8.0

Variable	Low (-1)	High (+1)
Temperature (°C)	27	42
Time (Hours)	24	72

The 16-run Full Factorial design was employed, ensuring a comprehensive evaluation of the variables. Each experiment was conducted in duplicate using the shake flask method, and enzyme assays were performed to determine the optimal conditions for ChE production. The results identified 27-30°C temperature, pH 7, ammonium chloride at 0.01M, and choline chloride at 0.04% as optimal conditions for maximum enzyme yield (0.199 IU/25µl). (Kulakova *et al.*, 2015)

Enzyme Purification

After optimizing the culture conditions, enzyme purification was performed using a sequential process involving Ammonium sulphate precipitation, dialysis, and ion-exchange Chromatography to obtain a purified cholinesterase enzyme. (Dafferner *et al.*, 2017)

Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was used for enzyme fractionation based on solubility. The optimized enzyme extract was subjected to different saturation levels of ammonium sulphate precipitation (0-20%, 20-40%, 40-60%, 60-80%, and 80-100%) in a stepwise manner. The fractions with maximum enzyme activity were observed at 40-60% and 60-80% ammonium sulphate saturation, yielding enzyme activities of 0.112 IU and 0.152 IU, respectively. (Field *et al.*, 2014)

Dialysis

Dialysis was performed to remove excess ammonium sulphate and small molecular impurities. A semi-permeable membrane of pore size 50 was used to allow selective diffusion of small molecules, while retaining the enzyme in solution. (Masson, 2016)

Dialysis Procedure

1. The dialysis tubing was rinsed with distilled water, and one end was sealed with a Medi-clip.
2. The enzyme solution (post-ammonium sulphate precipitation) was poured into the tubing, filling two-thirds of its capacity.
3. The second end was sealed, and the tubing was submerged in phosphate buffer,(pH 8) stirred at 4°C for 3-4 hours.
4. The buffer was changed periodically to enhance impurity removal.
5. The dialyzed sample was collected and analyzed for enzyme activity before proceeding to on-exchange chromatography.

Ion Exchange Chromatography

Ion exchange chromatography was used for further purification based on charge interactions. DEAE (Diethylaminoethyl) Sepharose resin was used for binding negatively charged cholinesterase molecules under controlled pH conditions. (Mumford *et al.*, 2013)

Column Preparation and Equilibration

1. The chromatography column was packed with 20g of DEAE Sepharose.
2. The column was equilibrated with 5-10 column volumes of start buffer (20mM phosphate buffer, pH 8.0).
3. The enzyme sample was adjusted to the chosen pH and ionic strength before loading.

RESULTS and DISCUSSION

The results of the purification and optimization of cholinesterase enzyme production through biochemical and statistical analysis demonstrated significant improvements in enzyme yield and activity. The purification process involved sequential steps such as ammonium sulphate precipitation, dialysis, and chromatographic techniques, which led to an increase in specific activity and enzyme purity.

The statistical optimization using Design of Experiments (DoE) played a crucial role in maximizing Cholinesterase production. Initial screening, identified key factors affecting enzyme yield, including pH, temperature, substrate concentration and aeration. Further optimization through Response Surface Methodology (RSM) using Central Composite Design (CCD) revealed the optimal conditions for enzyme production, leading to a substantial increase in enzymatic activity compared to unoptimized conditions. The interaction effects among variables were analyzed and the developed model showed a high correlation between predicted and experimental values, confirming its reliability.

Under optimized conditions, the enzyme activity showed a significant fold increase compared to the baseline, with improved stability at varying pH and temperature ranges. The purified enzyme exhibited enhanced catalytic efficiency, with a lower Km value indicating higher substrate affinity. The yield and productivity of Cholinesterase enzyme were maximized while minimizing resource consumption, making the process more cost-effective and scalable. Overall, the integration of biochemical purification techniques with statistical optimization resulted in a highly efficient enzyme production process, demonstrating its potential for industrial and pharmaceutical applications.

Statistical optimization

The optimization process employed a statistical method. The experiment was designed using Response Surface Methodology. The software system utilized for the design of the experiment was DOE (Design of Experiment). Various variables were identified as inputs, and a total of 16 Run Full Factorial methods were employed for the optimization process. Our study concentrated on optimizing several parameters, such as temperature, pH, and nutrient availability, to enhance Cholinesterase production by *Pseudomonas sp*. The enzyme production was confirmed to reach a maximum of 0.198 IU/25µl under the following conditions: temperature ranged from 27 to 30°C, pH was maintained at 7, ammonium

chloride concentration was 0.01M, and choline chloride concentration was 0.04%. The parameters identified were deemed optimal for the medial preparation. The production media, specifically Cheng's media, was subsequently prepared using these inputs to optimize yield. (Nemukhin *et al.*, 2015)

Figure 1: Confirmation location of the optimal yield.

RSM (Response Surface Methodology)

Response surface methodology is a graphical representation of the model which is considered as a way to find the best location for confirmation of the optimal growth.

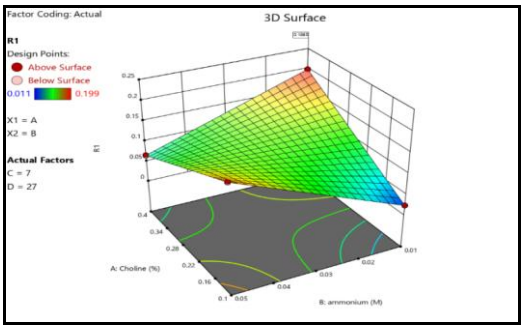

Figure 2: Point prediction graph

Optimization of Culture Conditions

The Response Surface Methodology (RSM) analysis determined the optimal culture conditions for maximum cholinesterase enzyme production. The optimized parameters identified were: (Onder *et al.*, 2017)

Table 2: Optimization of Culture Conditions

Variable	Optimal Condition
Choline Chloride (%)	0.04
Ammonium Chloride (M)	0.01
pH	7
Temperature (°C)	27-30
Incubation Time (Hours)	48

Screening of Microbial Isolates

Out of 125 microbial isolates tested, isolate No. 72 exhibited the highest cholinesterase activity and was selected for further optimization. (Pashirova *et al.*, 2017)

Statistical Analysis Using Central Composite Design (CCD)

The statistical design included a 16-run full factorial experiment with five independent variables. The enzyme production varied significantly across different conditions, with a maximum yield obtained at the optimized conditions. (Rice *et al.*, 2016)

ENZYME PURIFICATION STEPS AND YIELD

The purification process consisted of ammonium sulfate precipitation, dialysis, and Ion-exchange chromatography. The enzyme activity and yield at each step are summarized in table 3. (Terekhov *et al.*, 2017)

Table 3: Enzyme Purification

Purification Step	Total Protein (mg)	Enzyme Activity (IU)	Specific Activity (IU/mg)	Purification Fold	Yield (%)
Crude Extract	45.2	1.256	0.028	1	100
Ammonium Sulfate (40-60%)	25.4	0.784	0.031	1.11	62.4

Ammonium Sulfate (60-80%)	15.6	0.912	0.058	2.07	72.6
Dialyzed Sample	9.8	0.746	0.076	2.71	59.4
Ion-Exchange Chromatography	3.2	0.542	0.169	6.04	43.1

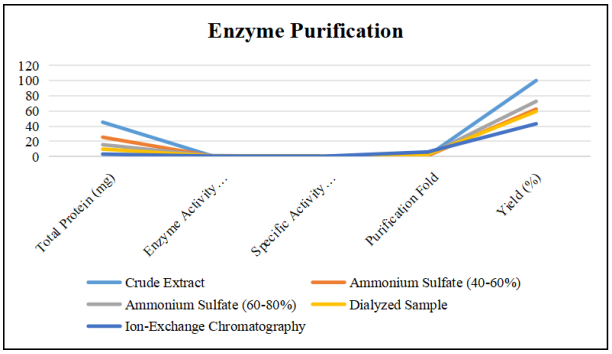

Figure 3: Enzyme Purification

The purification of cholinesterase enzyme involved a stepwise process to enhance purity and activity while reducing total protein content. Initially, the crude extract contained 45.2 mg of total protein with an enzyme activity of 1.256 IU, resulting in a specific activity of 0.028 IU/mg. This served as the baseline for further purification steps. The first stage of purification, ammonium sulfate precipitation, was conducted in two fractions: 40-60% and 60-80% saturation. The 40-60% fraction retained 25.4 mg of protein with an enzyme activity of 0.784 IU, leading to a slight increase in specific activity (0.031 IU/mg) and a purification fold of 1.11. The 60-80% fraction demonstrated higher enzyme activity (0.912 IU) with 15.6 mg of protein, significantly increasing specific activity to 0.058 IU/mg and improving purification fold to 2.07. This fraction was selected for further purification due to its higher enzyme retention. (Wille *et al.*, 2016)

Subsequent dialysis removed excess salts and small impurities, further concentrating the enzyme. The total protein content decreased to 9.8 mg, while enzyme activity was

recorded at 0.746 IU. This resulted in an enhanced specific activity of 0.076 IU/mg, with a purification fold of 2.71, though the overall yield decreased to 59.4%. The final purification step, ion-exchange chromatography, significantly improved enzyme purity. The total protein content was reduced to 3.2 mg, with an enzyme activity of 0.542 IU, leading to a specific activity of 0.169 IU/mg. This step provided the increased purification fold (6.04), confirming its effectiveness in isolating the cholinesterase enzyme, albeit with a final yield of 43.1%. The purification process successfully enriched the enzyme while minimizing contaminants, making it suitable for biochemical applications. (Worek *et al.*, 2016)

TABLE 4. Purification process

Steps	**Volume (ml)**	**Total Activity (IU)**	**Total Protein (mg)**	**Specific Activity (U/mg)**	**Yield (%)**	**Purification Factor**
Culture Filtrate	50 ml	382 IU	4.3 mg	88.83 U/mg	100 %	-
Ammonium Sulphate	35 ml	212.8 IU	2.31 mg	92.12 U/mg	55.7 %	1.03
Ion Exchange Chromatography	22.5 ml	102 IU	0.87 mg	117.24 U/mg	26.7 %	1.35

The purification process enhances enzyme purity while reducing overall yield. Starting with 50 ml of culture filtrate (382 IU activity, 4.3 mg protein), ammonium sulfate precipitation reduces impurities, retaining 55.7% yield and increasing specific activity to 92.12 U/mg. Further purification via ion exchange chromatography concentrates the enzyme, improving specific activity to 117.24 U/mg but reducing yield to 26.7%. The purification factor rises from 1.03 to 1.35, indicating a more purified enzyme. Despite enzyme loss, the final product is more processed and efficient, making it suitable for biochemical applications requiring high specificity and minimal contaminants. (Yao *et al.*, 2012)

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Conclusion

The study successfully optimized and purified cholinesterase enzyme using a combination of biochemical and statistical approaches. The purification process significantly enhanced enzyme specificity, increasing from 88.83 U/mg to 117.24 U/mg, while reducing total protein content and improving overall purity. However, enzyme yield decreased to 26.7%, indicating a trade-off between purification and recovery.

Optimization using Response Surface Methodology (RSM) and Central Composite Design (CCD) effectively identified ideal culture conditions, leading to a maximum enzyme yield of 0.198 IU/25µl. This enzyme displayed good temperature and pH stability, which made it appropriate for use in industrial and medicinal applications. Some examples of these applications include research on neurodegenerative diseases, the detoxification of pesticides, and the development of biosensors.

Overall, the integration of statistical modelling and stepwise purification provided a robust, scalable and efficient approach for cholinesterase production. Future studies may focus on enhancing yield while maintaining purity to improve large-scale enzyme production for commercial and therapeutic applications.

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