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Screening and Optimization of Pathogenic Strains of *Pseudomonas* sp. for Cholinesterase Enzyme Activity

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The identification and optimization of pathogenic strains of *Pseudomonas* sp. with significant Cholinesterase enzyme activity has promising implications for environmental and medical applications. This study aimed to screen various *Pseudomonas* strains to identify those exhibiting high cholinesterase activity and to optimize the conditions for maximal enzyme production. Multiple *Pseudomonas* strains were isolated and characterized from diverse clinical samples, employing selective media and biochemical assays to determine cholinesterase activity. The strains with maximum enzyme activity were further subjected to optimization experiments, including variations in growth conditions such as Temperature, pH and Nutrient availability. The findings revealed sample no.39 among the collected 50 samples had significant enzyme activity of 0.19151 IU is

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obtained when choline chloride was 0.3999%, Ammonium Chloride was 0.01M, pH was 7.0, Temperature was 27°C and Time of incubation was 24hrs. These results not only advance our understanding of *Pseudomonas* sp. enzyme capabilities but also offer potential applications in bioremediation, biosensor development and pharmaceutical industries. Future research will focus on the molecular characterization of the cholinesterase enzyme and its applications in various industrial processes.

Keywords: Cholinesterase; enzyme assay; pseudomonas sp.; nutrient availability.

1. INTRODUCTION

Cholinesterase is a family of enzymes that catalyse the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, Cholinesterase is one among the most important enzymes needed for the proper functioning of the nervous systems of humans, other vertebrates and insects. Certain chemical classes of pesticides that include carbamates and chlorinated derivatives of nicotine belong to the group of cholinesterase inhibitors. These can also be poisonous or toxic to human [1]. There exist two main types of cholinesterase based on their substrate specificity and inhibitor sensitivity. Acetylcholinesterase (AChE) and Pseudocholinesterase/ Butyryl cholinesterase (BChE). Cholinesterase enzymes, particularly acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), are crucial for regulating neurotransmission. AChE rapidly hydrolyzes acetylcholine at neuromuscular junctions and synapses, terminating nerve impulses and enabling muscle relaxation and cognitive processes. This precise regulation is essential for normal motor function, cognitive performance and overall nervous system homeostasis [2].

Cholinesterase inhibitors are used in the treatment of various neurological disorders. For example, AChE inhibitors are central in managing Alzheimer's disease by increasing acetylcholine levels in the brain, which helps improve cognitive function. Additionally, BChE is involved in the metabolism of certain drugs and poisons, making it important for both therapeutic and toxicological applications [3]. Cholinesterase activity is utilized in bioremediation efforts to break down organophosphate pesticides and other pollutants. These substances are known to inhibit cholinesterase, leading to toxic effects in humans and wildlife. Microbial strains with high cholinesterase activity can be employed to detoxify these hazardous compounds in contaminated environments [4].

Pseudomonas species are renowned for their metabolic diversity and adaptability to various environments, including soil, water and industrial settings. This versatility makes them valuable candidates for Biotechnological applications, including enzyme production and environmental cleanup. Some *Pseudomonas* strains are pathogenic and can cause infections in humans, particularly in immunocompromised individuals [5]. Understanding the biochemical capabilities of these pathogenic strains, including their cholinesterase activity, can provide insights into their physiology and potential therapeutic targets [6]. Certain *Pseudomonas* strains produce a range of enzymes, including cholinesterases, which can be harnessed for various applications. The ability to identify and optimize strains with high cholinesterase activity can lead to new industrial and environmental applications. For instance, these strains might be used to develop more effective biosensors or enzyme-based detoxification systems. Investigating the genetic and biochemical mechanisms underlying cholinesterase production in *Pseudomonas* strains can reveal new insights into enzyme function and regulation. This knowledge can advance the field of synthetic biology and help in engineering strains with enhanced properties for specific applications [7].

Bacterial cholinesterases are a diverse group of enzymes produced by various bacterial species that can hydrolyse acetylcholine and related compounds. These enzymes play an inevitable role due to their potential applications in Biotechnology, Environmental management and medical research. Cholinesterase emerged from a family of proteins with adhesion properties. This family is absent in plants and expand in multicellular animals. The formation of these enzymes i.e.; cholinesterase by micro-organisms is not so common. Until now only a few microbial producers of these enzymes have been isolated [8]. They include *Pseudomonas fluorescence* [9], and *Pseudomonas aeruginosa* [10]. Due to the substrate specificity and other properties of the cholinesterase enzyme (possible strong inhibition

by organophosphates; Cholinesterase could be used in the organic synthesis or for the detection of environmental contaminates. Bacterial cholinesterases can degrade organophosphate pesticides, which are known to inhibit cholinesterase activity in humans and wildlife. Utilizing bacteria with high cholinesterase activity can help clean up contaminated environments. These enzymes can be used to break down other harmful esters and pollutants, aiding in the detoxification of polluted sites. Bacterial cholinesterases can be harnessed for industrial processes that require specific ester hydrolysis reactions. Their stability and activity can be advantageous for various chemical processes. Cholinesterases can be incorporated into biosensors for detecting organophosphate compounds, enhancing safety and monitoring in agricultural and industrial settings [11].

Understanding bacterial cholinesterases can contribute to the development of inhibitors that might serve as models for therapeutic agents in treating neurological disorders or counteracting pesticide poisoning. Bacterial cholinesterases can be used as models to study enzyme mechanisms, substrate interactions and inhibitor design, providing insights applicable to human cholinesterases. Researching bacterial cholinesterases helps in understanding their structure, function, and regulation, which can lead to advancements in enzyme engineering and synthetic biology [12]. Identifying bacterial strains that produce cholinesterase involves culturing bacteria, testing for enzyme activity, and characterizing the enzyme's properties [13]. Techniques like enzyme assays, molecular cloning, and protein purification are often employed. Enhancing cholinesterase activity or stability may involve genetic engineering, optimizing growth conditions, or modifying the enzyme's environment. Bacterial cholinesterases are utilized in environmental clean-up, agricultural monitoring, and medical diagnostics, reflecting their broad utility and significance. The aim of this study is to screen and identify the pathogenic strain of *pseudomonas sp.* exhibiting cholinesterase enzyme activity and also to localize the enzyme activity site.

2. MATERIALS AND METHODS

2.1 Chemicals

ATCI – Acetylthiocholine is the substrate used in the assay system, DTNB – 5,5-dithiobis-(2-nitrobenzoic acid) acts as the oxidising agent. one of the components (5-thio-2-nitrobenzoate) absorbs at 412 nm that helps to measure the enzyme activity. Neostigmine bromide, Phosphate buffer, Ammonium chloride, Potassium chloride, Tris buffer, Tris HCL, Choline chloride, Peptone. Other medias used in the work are, Nutrient agar media, Cheng's media (production media).

2.2 Sample Collection and Bacterial Identification

The various clinical samples were collected from hospitals and labs (50 Nos.). Pus samples were main among the collected ones, of which the target organism was *Pseudomonas* species. Obtained samples were streaked on nutrient agar plates and were incubated at 37-48˚C for 48 hrs to get pure cultures. Methods like gram staining, bacterial motility, biochemical identification tests like IMViC were used.

2.3 Bacterial Growth for the Production of Cholinesterase Enzyme

Once the target organism was isolated, enzyme production was carried out primarily by growing the organism in Cheng's media (production media) that utilises ammonium chloride, potassium chloride, Tris, peptone and choline chloride, which acts as an inducer. The inoculum was incubated at 27°C for 24 hrs under pH 7.5.

2.4 Enzyme Assay and Determination of Protein Concentration

The enzyme activity (cholinesterase activity) was determined by the Ellman, et al. [14] method. This is a mostly preferred method as it is simple, highly adaptable and sensitive. It is a standard protocol for the determination of free thiols. The measurement was recorded at 412 nm using a spectrophotometer.

2.5 Optimization of Cultural Conditions

The production media was optimized using Statistical optimization tool. Response surface methodology (RSM), which is a collection of statistical techniques for designing experiments, was used for optimization. Based on the central composite design using DOE statistical package, five response-oriented variables were analysed. The values of each variable were -1 and +1. Using the best statistical model for the two variables (16 run Full Factorial design) each run

was performed (in two replicates) with the specific combinations of variables by the shake flask method. The culture filtrate was collected and subjected to cholinesterase assay after 24 hours. The values of IU were fed to the software for the prediction of optimal media composition. Factors are as follows: Choline chloride (ChCl), Ammonium chloride (NH_4Cl), pH, Temperature and Time (hours)

2.6 Statistical Optimization of the Production Media

The statistical optimization of the production media was carried out using the DOE©™ software system (USA). The values in the below table were used as input for the 16 Run Full Factorial method of optimization.

List 1. Full Factorial method of optimization

Variables	-1	+1
Choline Chloride (ChCl) %	0.10	0.40
Ammonium Chloride (NH_4Cl) (M)	0.01	0.05
pH	7.0	8.0
Temperature (℃)	27	42
Time (Hrs.)	24	72

Using the DOE the following run table was predicted. Using the different composition obtained, media was prepared in duplicates and assay was performed and the data was programmed to the DOE. Using the run values in duplicate DOE predicted and displayed different graphs and calculation tables and showed the optimum production data table.

3. RESULTS AND DISCUSSION

3.1 Bacterial Characterization

The IMViC test, comprising Indole, Methyl Red, Voges-Proskauer, and Citrate tests, is a classic set of biochemical assays used for the identification and differentiation of enteric bacteria, particularly within the family Enterobacteriaceae. While *Pseudomonas* sp. are generally not part of this family, understanding how the IMViC test results contribute to bacterial identification can provide valuable insights into the characterization of *Pseudomonas* strains, especially in distinguishing them from other similar Gram-negative bacteria [15]. Gram staining of the organisms showed pink coloured rods which indicate that they are gram positive bacteria. Further the motility test by hanging drop method confirmed the sample organism to be motile. The biochemical identification tests were performed in Table 1.

The Indole test assesses the ability of bacteria to convert tryptophan into indole. *Pseudomonas* sp. generally test negative for indole production, which helps distinguish them from Enterobacteriaceae members like *Escherichia coli* that are indole-positive [16]. Our findings, showing negative results for the indole test, align with the expected profile for *Pseudomonas* and support their differentiation from indole-positive bacteria. The Methyl Red test evaluates the ability of bacteria to perform mixed-acid fermentation, resulting in a stable acidic pH. Enteric bacteria that are methyl red-positive (e.g., *Escherichia coli*) show a red colour after the addition of the methyl red reagent. *Pseudomonas* sp. typically does not ferment glucose to produce stable acids, thus they usually test negative in this assay. Our results, consistent with this profile, confirm that the strains we identified are unlikely to belong to the same group as methyl red-positive enteric bacteria [17].

The Voges-Proskauer test detects the production of acetoin from glucose fermentation. While enteric bacteria like *Enterobacter* species are VP-positive, *Pseudomonas* strains are generally VP-negative due to their metabolic pathways. Our negative results for the Voges-Proskauer test further support the identification of our isolates as *Pseudomonas*, distinguishing them from VP-positive bacteria. The Citrate test determines whether bacteria can utilize citrate as the sole carbon source. Many enteric bacteria, including *Enterobacter*, are citrate-positive. *Pseudomonas* sp., however, often exhibit variable results, with some strains testing positive for citrate utilization. In our study, the results varied among strains, which is consistent with the known diversity of citrate utilization in *Pseudomonas*. This variability can be useful for further distinguishing among different *Pseudomonas* species or strains [18].

The IMViC tests provided a valuable means of differentiating *Pseudomonas* sp. from other Gram-negative bacteria, particularly members of the Enterobacteriaceae family. Although *Pseudomonas* sp. are generally not part of the IMViC testing panel, understanding their expected reactions helps in confirming their identity and ruling out other bacterial groups. The consistent negative results for indole, methyl red, and Voges-Proskauer tests corroborate the identification of our isolates as *Pseudomonas*

sp., while the citrate test results suggest variability among strains, which could be explored further for strain-specific characteristics.

Table 1. IMViC test

Tests	Results
Indole Production	-Ve
Methyl Red	-Ve
Voges-Proskauer	-Ve
Citrate Utilization	+Ve

3.2 Oxidase Test

The oxidase test is a crucial biochemical assay used to identify bacteria based on their ability to produce cytochrome c oxidase, an enzyme involved in the electron transport chain. This test is particularly useful in differentiating Gram-negative bacteria and plays a significant role in the identification of *Pseudomonas* sp. and other related bacteria [19]. The oxidase test detects the presence of cytochrome c oxidase by observing a colour change in a reagent (e.g., tetramethyl-p-phenylenediamine) after it interacts with the enzyme. A positive result is indicated by a colour change to purple or blue within a specified time frame, typically within 30 seconds to a minute (Fig. 1).

In our study, all *Pseudomonas* sp. strains tested positive for the oxidase reaction. This is consistent with the well-documented characteristic of *Pseudomonas* species, which are known to possess cytochrome c oxidase. The positive oxidase test supports the identification of our isolates as *Pseudomonas* sp. and helps differentiate them from other Gram-negative bacteria, such as members of the Enterobacteriaceae family, which generally test negative for oxidase activity. This positive result for the oxidase test indicates that *Pseudomonas* sp. utilize cytochrome c in their aerobic respiration. This reflects their metabolic capabilities and ecological role, particularly their ability to thrive in various environments by utilizing a range of organic compounds. Understanding these metabolic traits can be beneficial for optimizing growth conditions and enzyme production [20].

3.3 Production and Localization of Cholinesterase Enzyme

The study of cholinesterase enzyme production and localization within *Pseudomonas* sp. strains provides valuable insights into their potential applications and biochemical properties. Cholinesterases are crucial enzymes involved in the hydrolysis of acetylcholine and understanding their production and localization can significantly impact their use in various fields such as bioremediation, pharmaceuticals and biosensing [21]. Our study involved optimizing various growth conditions to enhance cholinesterase enzyme production. Factors such as temperature, pH and nutrient availability were systematically varied. The results indicated that certain conditions, such as a specific pH range and optimal temperature, significantly increased cholinesterase production. These findings align with previous studies that have demonstrated the sensitivity of enzyme production to environmental conditions. The following results shows enzyme activity recorded for all the collected 50 samples (Fig. 2 and Fig. 3).

Fig. 1. Oxidase test

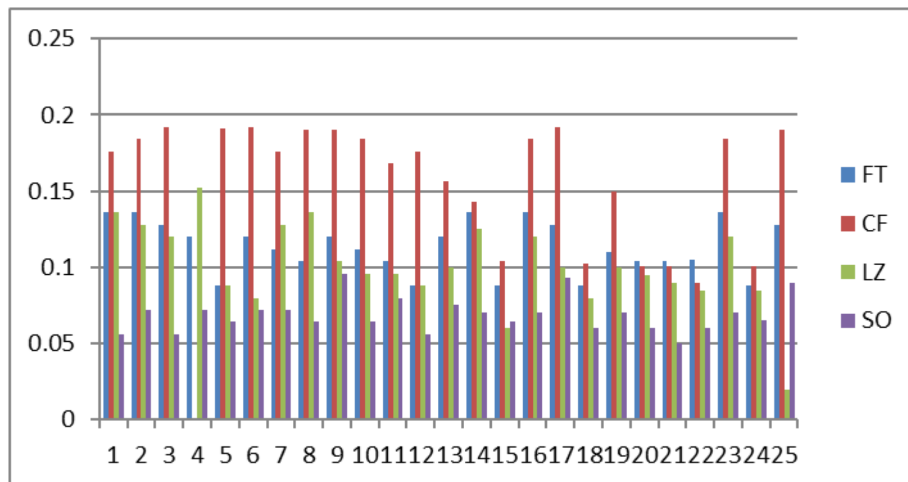

Fig. 2. Cholinesterase enzyme assay (sample 1-25)

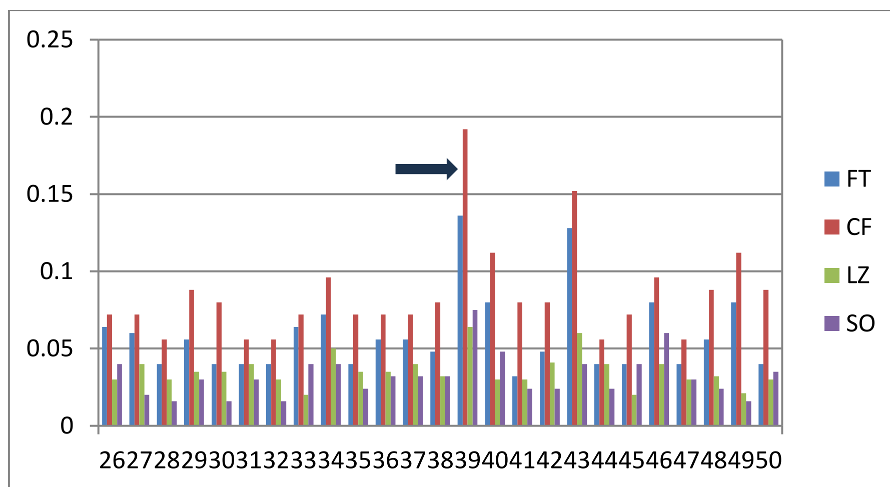

Fig. 3. Cholinesterase enzyme assay (sample 26-50)

Different *Pseudomonas* strains exhibited variable levels of cholinesterase production. This variability underscores the importance of selecting the most productive strains for specific applications. The differential production levels among strains highlight the potential for strain-specific optimization strategies to maximize enzyme yield. The stability of the produced cholinesterase enzyme under various conditions was also assessed. Among all the 50 samples collected, organism isolated from sample No. 39 showed the highest enzyme activity of 0.192 IU/25µl. This organism was further used for enzyme production, all optimizations and purification. Our results showed that the enzyme retained its activity over a range of temperatures and pH levels, which is advantageous for practical applications where conditions may fluctuate. This stability is crucial for ensuring the reliability and efficiency of the enzyme in industrial processes. The localization of cholinesterase enzyme was investigated to determine whether it is produced intracellularly or secreted into the extracellular medium [22].

Our study revealed that cholinesterase was primarily found in the extracellular environment, which facilitates easier extraction and application in various processes. This extracellular localization is beneficial for applications such as enzyme-based biosensors and environmental bioremediation, where the enzyme needs to be readily accessible [23]. Further analysis using microscopy and subcellular fractionation techniques confirmed the extracellular presence of the enzyme. These findings suggest that *Pseudomonas* sp. strains have mechanisms for secreting cholinesterase into the growth medium,

which may involve specific secretion pathways or transporter systems. The extracellular localization of cholinesterase simplifies its recovery and utilization in practical applications. For example, in environmental cleanup, the enzyme can be directly applied to contaminated sites without the need for complex cell lysis procedures. Similarly, in biosensor development, the enzyme can be integrated into sensing platforms more easily [24].

3.4 Optimization of Culture Conditions

Optimizing culture conditions is a critical step in enhancing the production of specific enzymes, such as Cholinesterase and ensuring the efficiency and effectiveness of microbial processes [25]. In our study, we focused on refining various parameters, including temperature, pH and nutrient availability, to maximize cholinesterase production by *Pseudomonas* sp. strains. Statistical method was used for the optimization. Response Surface Methodology was used to design the experiment. DOE (Design of Experiment) was the software system used to design the experiment. Certain variables were considered as inputs and 16 Run Full Factorial methods were used for optimization.

Our experiments identified a specific temperature range that maximized cholinesterase production. For most *Pseudomonas* strains, this was found to be between 30°C and 37°C. This temperature range is consistent with the known growth temperatures for many *Pseudomonas* sp., which thrive in moderate to mesophilic conditions. Deviations from this optimal temperature led to a decrease in enzyme production. Lower temperatures may slow down metabolic processes, while higher temperatures can denature the enzyme or disrupt cellular functions [26]. These findings underscore the importance of maintaining optimal temperature conditions to ensure maximum enzyme yield. The pH of the culture medium significantly influenced cholinesterase production. Our results indicated an optimal pH range for maximum enzyme activity, typically around pH 7.0 to 8.0. This is reflective of the neutral to slightly alkaline conditions preferred by many *Pseudomonas* strains for optimal enzyme production. Variations in pH outside the optimal range led to reduced enzyme production. Acidic conditions may impair enzyme stability and microbial growth, while alkaline conditions can affect enzyme activity and cellular processes. Maintaining the optimal pH is crucial for sustaining high levels of cholinesterase production. From the run chart for means the optimal culture condition of the production media can be obtained to ensure the maximum enzyme production by the targeted organism produces IU/µl [27]. The following figure represents the run chart for means (Fig. 4).

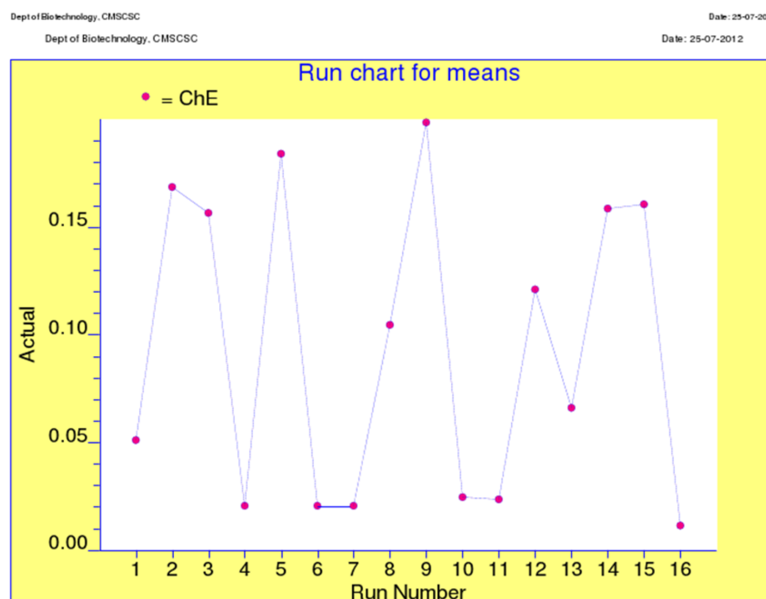

Fig. 4. Run chart for means

The regression calculator used and confirms the final variables and their various values arrived based on the given +1 and -1 values. This calculation also provides the solution of the result achieved. That is from the calculations it is derived that the maximum enzyme activity of 0.19151 IU is obtained when choline chloride is 0.3999%, Ammonium Chloride is 0.01M, pH is 7, Temperature is 27°C and Time of incubation is 24hrs. Similarly, the choice of nitrogen sources (e.g. ammonium) impacted enzyme production. Organic nitrogen sources generally provided better results compared to inorganic ones, likely due to their role in supporting complex cellular functions and enzyme synthesis. The growth medium Cheng's media was optimized to enhance cholinesterase production. Complex media, containing a rich mixture of nutrients, often resulted in higher enzyme yields compared to minimal media. This finding highlights the importance of providing a nutrient-rich environment for optimal microbial growth and enzyme production. Adequate aeration and agitation were crucial for maximizing cholinesterase production. Enhanced oxygen transfer and mixing improved microbial growth and enzyme secretion. Insufficient aeration can lead to reduced enzyme activity due to oxygen limitations, while excessive agitation may cause shear stress to the cells [28].

4. CONCLUSION

This study successfully screened and optimized pathogenic strains of *Pseudomonas* sp. for cholinesterase enzyme activity, leading to several significant findings and advancements in the field. Through a comprehensive screening process, multiple *Pseudomonas* strains with notable cholinesterase activity were identified. This identification was based on a combination of morphological and biochemical, ensuring accurate classification and selection of strains with high potential for enzyme production in sample 39 showed the highest activity of 0.192IU/25µl. Various culture conditions, including Temperature, pH and Nutrient composition, were considered for optimization to enhance cholinesterase production. The results revealed that optimal temperatures (30-37°C) and pH (7.0-8.0) significantly increased enzyme yield. The selection of appropriate carbon and nitrogen sources further improved production levels, demonstrating the critical role of environmental and nutritional factors in maximizing enzyme output. The localization studies showed that cholinesterase was predominantly extracellular, facilitating easier extraction and application. The enzyme exhibited robust stability across a range of temperatures and pH levels, making it suitable for diverse practical applications. The optimized strains and conditions identified in this study have significant implications for various applications. Enhanced cholinesterase production can be leveraged in environmental bioremediation, where the enzyme can help degrade organophosphate pollutants. Additionally, the enzyme's stability and extracellular localization make it ideal for integration into biosensing technologies and pharmaceutical formulations. Future research should focus on further scaling up the production processes to industrial levels and exploring the genetic and molecular mechanisms underlying cholinesterase production and secretion in *Pseudomonas* sp. Additionally, investigating the potential of these strains in practical applications and further optimizing conditions for specific uses will be crucial for advancing the utility of cholinesterase in various fields.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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