International Journal of Design & Nature and Ecodynamics
Vol. 20, No. 6, June, 2025, pp. 1343-1349
Journal homepage: http://iieta.org/journals/ijdne

Bioremediation Potential of *Pseudomonas aeruginosa* Protease: Efficient Degradation of Diesel Hydrocarbons Through Purified Enzyme Application

Wafaa Hassan Muslem[1*], Sahira Nsayef Muslim[1], Huda Zuheir Majeed[1], Hassan Muslem Abdulhussein[2]

[1] Department of Biology, College of Science, Mustansiriyah University, Baghdad 10064, Iraq
[2] Radiology Techniques Department, Dijlah University College, Baghdad 10064, Iraq

Corresponding Author Email: wafaahassan2017@uomustansiriyah.edu.iq

https://doi.org/10.18280/ijdne.200614

Received: 1 June 2025
Revised: 22 June 2025
Accepted: 25 June 2025
Available online: 30 June 2025

Keywords:
Pseudomonas aeruginosa, diesel degradation, protease

ABSTRACT

Global concern over diesel pollution is growing, particularly with regard to the marine environment. To remove this pollution, a number of methods have been used; however, most of them are not eco-friendly. An economical, efficient, and ecologically beneficial technical method for treating diesel contamination and eliminating hazardous hydrocarbons from contaminated environments is biodegradation. Thus, the primary goal of this investigation was to assess the activity of a protease that has the capacity to degrade diesel hydrocarbons. *Pseudomonas aeruginosa* strain W4 was isolated from wound and burn infections and selected for its superior protease production. Further criteria for selection included its high enzymatic activity on casein agar and its ability to thrive under various environmental conditions relevant to bioremediation. Protease synthesis was initially detected using a main technique involving a nutrient agar plate with casein (1% w/v) as a substrate, with secondary quantification showing a robust activity of 33 U/mL. The purification process, yielding a final 32% recovery, involved ammonium sulfate precipitation, followed by DEAE-cellulose column chromatography using a phosphate buffer at pH 8 to optimize protein binding and elution, and finally a Sephadex G-100 column. The purified protease had a molecular weight of 50 kDa. Microbial protease significantly aided hydrocarbon breakdown. While direct enzyme application resulted in 34% diesel breakdown, the proportion of hydrocarbon degradation remarkably rose to 79% after 25 days as enzyme concentration and incubation time increased. This demonstrates a 132% improvement in degradation efficiency compared to the initial direct application. Consequently, this highly efficient and eco-friendly enzyme can be effectively used in petroleum-contaminated locations as a potent bioremediation agent, offering a sustainable alternative to traditional remediation methods.

1. INTRODUCTION

Global concern over diesel pollution is escalating, posing significant threats to ecosystems, particularly marine environments. Recent reports underscore the urgency of addressing this issue. For instance, the International Maritime Organization (IMO) 2023 report highlights persistent challenges in controlling oil spills and emissions from shipping, with diesel components being a major contributor to marine hydrocarbon contamination worldwide. Locally, Iraq faces a pressing challenge with diesel pollution, especially in areas affected by industrial activities, oil extraction, and transportation. Unregulated disposal, leaks from pipelines, and inefficient combustion processes contribute to widespread soil and water contamination in regions like Basra and Kirkuk. This local context underscores an urgent need for effective, sustainable, and environmentally friendly remediation strategies. To combat this widespread pollution, various bioremediation methods have emerged as eco-friendly alternatives to traditional physical and chemical approaches. These methods typically involve the use of microorganisms or their components to degrade pollutants. Broadly, bioremediation approaches can be categorized into whole-cell bioremediation (using intact microorganisms) and enzyme-based bioremediation (using purified enzymes). While whole-cell approaches, such as those employing *Pseudomonas aeruginosa*, are effective in degrading petroleum hydrocarbons (PHCs) due to their diverse enzymatic machinery and biosurfactant production, they carry inherent risks. These risks include the potential for uncontrolled microbial growth, the release of metabolic byproducts, and, critically, the spread of opportunistic pathogens into the environment, especially when dealing with strains like *P. aeruginosa*, which is a known human pathogen. This is where the use of purified enzymes offers significant advantages. By utilizing isolated and purified enzymes, such as proteases, the risks associated with whole-cell applications are mitigated. Pure enzymes offer higher specificity for target pollutants, allowing for more controlled degradation processes. They also avoid the proliferation and spread of the microbial host,

thereby eliminating concerns regarding pathogenicity or ecological disruption. Furthermore, purified enzymes can be applied under a wider range of environmental conditions (e.g., pH, temperature, salinity) than living cells, and their activity can be more precisely controlled and optimized.

Pseudomonas aeruginosa, a Gram-negative bacterium, is widely recognized for its metabolic versatility and ability to degrade various organic compounds, including hydrocarbons. It produces a range of virulence factors, among which proteases play a crucial role in its physiological processes. These proteases, including alkaline protease and elastases, have long been studied for their diverse industrial applications in detergents, pharmaceuticals, and food processing. More recently, their potential in bioremediation has gained significant attention due to their ability to hydrolyze complex organic molecules.

Hydrocarbon petroleum, primarily composed of saturated and aromatic hydrocarbons, represents a complex mixture that is challenging to remediate. The ability of *P. aeruginosa* to degrade PHCs has been linked to its possession of specific genes and the secretion of functional enzymes (e.g., alkane hydroxylase, dehydrogenase, catalase, and lipase), alongside its production of biosurfactants like rhamnolipid. Given the unique characteristics of different *P. aeruginosa* strains, often varying by their habitat of isolation and sensitivity to nutrients. This study focuses on harnessing the enzymatic power of this bacterium. The current study aims to screen and purify a specific protease from *P. aeruginosa* and meticulously examine its effectiveness as an efficient and safer agent for the bioremediation of diesel-contaminated environments, providing a targeted and environmentally sound solution to a pressing global and local challenge.

Pseudomonas aeruginosa is an opportunistic human pathogen that causes significant morbidity and mortality in patients suffering from eye disease, cancer, acquired immunodeficiency syndrome, burns, cystic fibrosis, pneumonia, urinary tract infections, and skin infections [1, 2]. With a 70% fatality rate, *P. aeruginosa* is the most frequent pathogen linked to infections in intensive care units and the primary cause of nosocomial pneumonia [3, 4]. Gram-negative *P. aeruginosa* is an opportunistic human pathogen that causes infections of the cornea, burn wounds, and urethra. It also exacerbates a number of illnesses, including pneumonia and cystic fibrosis [1, 5]. Toxins, phenazines, pyocyanin, and proteases are some of *P. aeruginosa'*s virulence factors that are essential to the infections [5, 6].

When *P. aeruginosa* infections produce tissue injury, proteases play a crucial role. Alkaline protease and two elastases (A and B) secreted by *P. aeruginosa* have been identified as exoenzymes and virulence factors [4, 5]. Elastase synthesis is correlated with *P. aeruginosa* invasiveness in burn patients [6]. For many years, the protease enzyme has been used extensively in a wide range of industries, including detergents, pharmaceuticals, cosmetics, leather, peptide synthesis, photography, textiles, agriculture, synthetic biotechnology, and the food industry, including baking, meat tenderization, brewing, and cheese production [7]. Furthermore, the protease enzyme is used in many medical sectors for both people and animals, including various medical diagnoses, particular medical treatments for inflammation, malicious wounds, and sheepskin unhairing, as well as various bioremediation procedures [7, 8].

Protein molecules can be hydrolyzed into amino acids by proteases, which are extracellular enzymes that microbes can use [6]. Additionally, they have been regarded as the ideal sources for producing the protease enzymes due to their wide variety of microorganisms, quick and high growth rates, affordability, environmental friendliness, ease of troubleshooting, minimal cultivation space, and versatility in genetic manipulation levels [5, 9].

Diesel is a popular fuel for both electric power generation and transportation [10]. This blend of hydrocarbons includes monoaromatic, polyaromatic, and saturated hydrocarbons. Diesel has a higher boiling point than gasoline and a lower boiling point than gas oil [11]. Diesel (hydrocarbon petroleum) is made of petroleum-derived hydrocarbons, with a 75% saturated hydrocarbon content that includes paraffins and a 25% aromatic hydrocarbon content that includes naphthalenes and alkyl benzenes. With a range of roughly $C_{10}H_{22}$ to $C_{15}H_{32}$, its typical chemical formula is $C_{12}H_{26}$ [12]. The cost-effectiveness and environmental sustainability of using microorganisms to remediate petroleum hydrocarbon (PHC)-contaminated areas appeal to both researchers and policymakers [12, 13]. It has been shown that the bacterial genus *P. aeruginosa*, which is frequently present in soil and water, degrades PHC [13]. In relation to the bioremediation of PHC-contaminated locations, it has several associated genes that secrete a variety of functional enzymes (e.g., alkane hydroxylase, dehydrogenase, catalase, and lipase), and creates the biosurfactant rhamnolipid [13, 14]. Furthermore, *P. aeruginosa* exhibits phenotypic differences due to its high sensitivity to nutrients, which differs from strain to strain [12, 14]. Additionally, under the same growth conditions, *P. aeruginosa* strains isolated from various habitats have distinct characteristics. The current study has concentrated on the goal of screening and purifying *P. aeruginosa* protease and examining its effectiveness in diesel used for environmental pollution remediation.

2. MATERIALS AND METHODS

2.1 Isolation and identification of *Pseudomonas aeruginosa*

50 swab specimens were taken from burn and wound samples were collected from local two hospitals: Al-Kindi Hospital and the Teaching Hospital of Baghdad Medical City, between April and July of 2024. The sample size for isolation was determined based on the prevalent hospital infection rate data in the region, aiming to ensure a representative collection of *Pseudomonas aeruginosa* strains associated with clinical settings. Using a transport media swab, the samples were moved into the cool box and incubated at 4℃. For the purpose of isolating *Pseudomonas aeruginosa*, the bacterial swabs were cultivated in brain heart infusion broth, incubated for 24 hours at 37℃, and then inoculated into selective media such as MacConkey agar and cetrimide agar. The characteristic morphology, pigment production, and confirmed using Gram staining, oxidase test, and standard biochemical tests, as well as growth at 4℃ and 42℃, are used to identify bacterial isolates [15, 16]. The Vitek-2 small instrument verified the grown isolates.

2.2 Primary and secondary screening for protease production

Protease synthesis in *Pseudomonas aeruginosa* was investigated for qualitative screening. On a nutrient agar plate

with casein (1% w/v) as a substrate, a loopful culture of *Pseudomonas aeruginosa* was streaked. Enzyme activity was measured after the plates were incubated for 24 hours at 37°C. Using the method outlined by study [17], the presence of a clear zone of hydrolysis on casein agar indicated the presence of proteolytic activity. The chosen bacterial isolates were cultured for 24 hours at 37°C after being injected in nutritional broth that contained casein (1% w/v). The cultures were centrifuged at 10,000 RPM for 15 minutes at 4°C, and the supernatant was removed [18].

2.3 Protease assay and protein concentration

The method described by study [19] was used to measure the protease activity in crude enzyme extract using casein as a substrate. A 0.65% casein solution was made in 0.1M Tris-HCl buffer (pH 8.5). Glass vials containing 5 mL of 0.65% casein solution were placed in an oven set at 37°C for 5 to 10 minutes. In each vial, one milliliter of crude enzyme was added. After giving it a slight shake to homogenize it, it was baked for 30 minutes at 37°C. By adding 5 mL of cold Trichloroacetic acid (TCA 10%), the reaction was stopped. The solution from each vial was then sieved through 0.45 µm syringe filters after the vials were allowed to sit at room temperature for half an hour. A second vial containing 2 mL of filtrate was then taken. This filtrate was mixed with five milliliters of 0.5 M Na_2CO_3 in each vial. Immediately following Na_2CO_3, one milliliter of Follin's Ciocalteus reagent (diluted by two times) was added. The final answer was put somewhere dark. At 680 nm, the absorbance value was calculated. The quantity of enzyme needed to hydrolyze casein and release the equivalent of one microgram of phenolic amino acids in a minute was considered one unit of enzyme activity. The Bradford method was utilized to quantify the protein concentration, with the standard protein being the protein found in bovine serum [20].

2.4 Purification of protease

Protease purification was carried out using a modified version of study [21]. Ammonium sulfate was added in varying quantities (20–80%) to precipitate the cell-free solution. The supernatant precipitated on its own when the concentration of $(NH_4)_2SO_4$ was raised by 10%. At 4°C, the enzyme solutions were shaken for one hour. The precipitates from the ammonium sulfate saturation level at which the most protein precipitated were gathered by centrifugation for 15 minutes at 10,000 rpm and 4°C. To create the concentrated enzyme suspension, the precipitates in pellet form were dissolved in 25 milliliters of 0.1 M Tris-HCl buffer with a pH of 8. The enzyme suspension was dialyzed in a dialyzing membrane in the same buffer at 4-8°C in order to eliminate the ammonium sulphate ions. Following equilibration with the same buffer, the protein sample was put onto the DEAE-Cellulose Anion Exchange Chromatography. Protein was eluted from the column using a gradient of 0.1 M to 1 M NaCl. The fractions were collected at a flow rate of 1 milliliter per minute. Using a UV Vis Spectrophotometer, the optical density (OD) of each percent was measured at 280 nm. After loading the active fractions onto a Sephadex G-100 column, the fractions were combined with the same. For additional research, the active portions were gathered and concentrated.

2.5 SDS-PAGE analysis

Following the procedure described in study [22] and using a tiny piece of glass plates (8 × 8 cm) gel apparatus, a 12% SDS-PAGE was carried out. Standard molecular weight markers were used to determine the molecular weights of the purified, crude, and pelleted ammonium sulphate precipitation.

The same quantities of purified protease in varying concentrations (600, 300, 150, and 75 µg/ml) were combined with 6% diesel fuel and incubated for 24 hours at 30°C. As a control, distilled water was utilized instead of the protease. To find out how many hydrocarbons remained after interacting with pure protease, the same amounts of mixture and toluene were mixed and compared to the control. Following mixing, the absorbance at 410 nm of the toluene with hydrocarbon dissolved in it was measured [23]. The following formula was used to determine the percentage of hydrocarbon degradation: (optical density for control-optical density for test) / optical density for control × 100. The same experiment was conducted again, but this time the ideal concentration of purified protease was used. The same amounts were taken every five days for a month, and the percentage of hydrocarbon degradation was calculated.

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of *Pseudomonas aeruginosa*

The results demonstrated that only 19 *Pseudomonas aeruginosa* isolates out of 50 samples taken from diverse clinical sites (burns and wounds) of patients exhibited typical morphological characteristics and biochemical tests that were consistent with *Pseudomonas aeruginosa*, whereas the other isolates were consistent with other infectious bacteria. A positive result for *P. aeruginosa* is indicated by the presence of green color on cetrimide agar, which demonstrates the bacteria's capacity to manufacture pyocyanin pigment. With the exception of *P. aeruginosa*, bacterial growth is inhibited by cultivation on Cetrimide agar (N-acetyl-NNN-trimethyl-ammonium-bromide). With the exception of *P. aeruginosa*, it functions as a quaternary structure cationic ammonium cleaner, which releases nitrogen and phosphorus from bacterial strains [24]. *P. aeruginosa* was the most isolated organism from wound and surgical site infections. One of the major contributors to nosocomial infections and community-acquired diseases is this organism. Geographically and within hospital units, *P. aeruginosa*'s prevalence and pattern of antibiotic sensitivity differ [25].

3.2 Primary and secondary screening for protease production

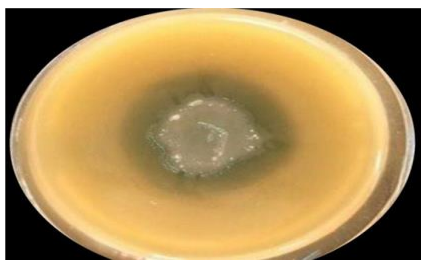

Figure 1. Protease production in nutrient agar plate with casein (1% w/v) in primary screening

Out of the 19 bacterial isolates that were identified as belonging to *P. aeruginosa*, only 16 isolates (84%) showed positive results for producing protease with a clear zone diameter of 14–28 mm, as shown in Figure 1. Additionally, 3 isolates (16%) showed negative results for protease after being cultured in skim milk agar. After secondary analysis, the *P. aeruginosa* producer isolates, as shown in Table 1, *P. aeruginosa* produced the protease at varying concentrations, with *P. aeruginosa* W4 producing 33 U/mL.

Table 1. Detection of protease production from *P. aeruginosa* by primary and secondary screening

Isolate	Diameter of Clear Zone (mm)	Protease Activity (U/mL)
P. aeruginosa W1	-	
P. aeruginosa W2	14	20
P. aeruginosa W3	22	25
P. aeruginosa W4	28	33
P. aeruginosa W5	17	22
P. aeruginosa W6	-	
P. aeruginosa W7	26	25
P. aeruginosa W8	23	30
P. aeruginosa W9	20	26
P. aeruginosa W10	-	
P. aeruginosa W11	25	29
P. aeruginosa W12	22	23
P. aeruginosa B1	23	26
P. aeruginosa B2	25	28
P. aeruginosa B3	21	25
P. aeruginosa B4	18	22
P. aeruginosa B5	18	21
P. aeruginosa B6	20	24
P. aeruginosa B7	19	22

Our findings concurred with study [26], which verified that the proportion of isolated *P. aeruginosa* that tested positive for exotoxin A and the protease test was greater than 95%. Two classes can be distinguished by the phase of the cell development cycle where the synthesis of extracellular protease peaks: (i) the rate of extracellular protease secretion is very low during the active growth phase and then increases during the late exponential and early stationary phases of growth; (ii) extracellular enzyme synthesis and secretion rise with growth and fall as the culture enters the stationary phase. Studies [27, 28] found that *P. aeruginosa*'s pathogenicity was linked to a number of extracellular components, including alkaline protease and elastase, and that these enzymes contributed to tissue damage and bacterial invasion during infection. Additionally, it was reported that *P. aeruginosa*'s extracellular protease production is influenced by nutritional factors such as carbon sources. This means that growth and protease production increased proportionately with increasing glucose concentration up to 0.07M, but these results declined at higher concentrations and in the absence of glucose (both growth and protease production were poor) [29].

3.3 Purification of protease

When ammonium sulfate saturation reached 70%, the crude protease precipitated with strong activity. The concentrated enzyme solution was run over a DEAE-cellulose column following the dialysis process. Three protein peaks emerged following the elution stage, and the protease activity found in the third protein peak was specific activity and recovery percentage, as shown in Figure 2. After being combined, the active fractions were run through a Sephadex G-100 column. One protein peak with protein activity U/mL and yield percentage appeared as a result of the elution step, as shown in Figure 3 and Table 2.

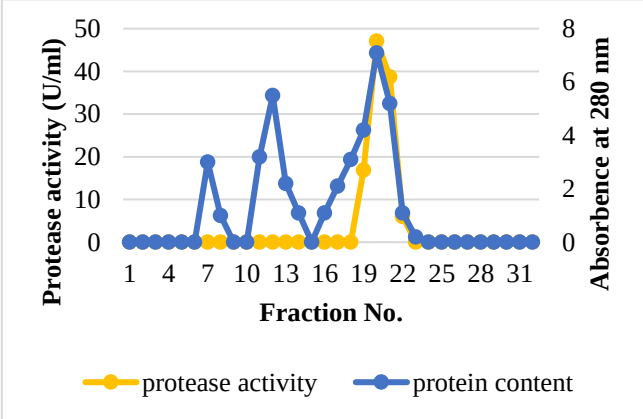

Figure 2. Purification of protease from *P. aeruginosa* by DEAE-cellulose column

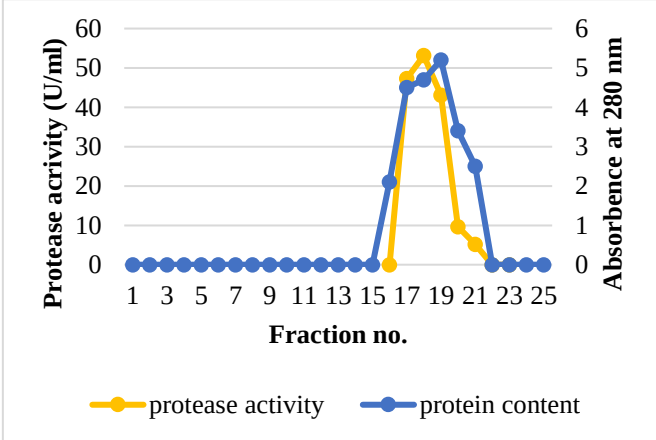

Figure 3. Purification of protease from *P. aeruginosa* by Sephadex G-100 column

Table 2. Steps of protease purification from *P. aeruginosa*

Purification Step	Crude Extract	$(NH_4)_2SO_4$ Precipitation	DEAE-Cellulose	Sephadex G-100
Size (mL)	80	45	20	16
Protease activity (U/mL)	33	41	47	53
Protein Conc. (mg/mL)	3.87	295	2.05	1.26
Specific activity (U/ mg)	8.52	13.89	22.29	42.06
Total activity	2640	1845	940	848
Purification fold	1	1.63	2.61	4.93
Yield (%)	100	69.8	35.6	32

Ion exchange chromatography using DEAE-Celluose and gel filtration on Sephadex G-75 was used to purify an alkaline protease from *Pseudomonas aeruginosa* MS71, yielding total purification folds of 10.87% and 13.1% recovery [30]. To purify the protease from Bacillus subtilis, Sephadex G-50 and CM-Sephadex chromatography were employed [31].

3.4 SDS-PAGE analysis

Figure 4 shows the isolated protease on a 12% SDS PAGE. Molecular weight indicators indicated that the protein weighed 50 kDa.

An extracellular proteolytic enzyme with a molecular weight of 18 kDa was generated by *P. aeruginosa* [32]. According to the protease IV monomer's mobility on SDS-PAGE under reducing circumstances and its elution in a single symmetrical peak on gel filtration, its estimated molecular mass is around 30 kDa [33].

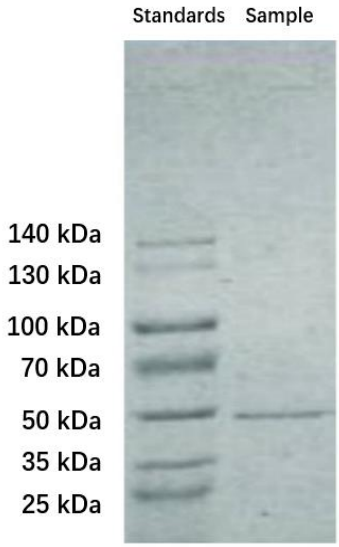

Figure 4. SDS-PAGE analysis for purified protease from *P. aeruginosa*

3.5 Determination of diesel degradation

After combining the hydrocarbon-diesel fuel with the purified protease, the absorbency of toluene was assessed. As shown in Figure 5, the results showed that raising the protease concentration increased the rate of hydrocarbon-diesel fuel degradation to 34% after 24 hours at 600 µg/mL. As seen in Figure 6, the hydrocarbons were broken down at a greater rate when the incubation period was extended. This degradation peaked after 25 days and then began to decline. Consequently, bioremediation has gained recognition as a substitute technique for cleaning up regions contaminated by hydrocarbons. Using this technique, the microbial metabolites can be used to hydrocarbon bioremediate the contaminated areas and transform these pollutants into chemicals of interest.

Many different cleanup techniques have been used, including pricy conventional techniques that use a lot of power and huge machinery [34]. As an alternative, enzyme-based bioremediation is a simple, sustainable, and environmentally beneficial method. Enzymes are described as bioremediation agents that speed up hydrocarbon biodegradation in reports from the US Environmental Protection Agency (EPA) [35]. Numerous elements, such as water activity, temperature, salinity, pressure, and oil content, influence how quickly oil degrades [35, 36]. Numerous elements, such as water activity, temperature, salinity, pressure, and oil content, influence how quickly oil degrades [36]. The ability of the enzymes lipases, dehydrogenases, catalases, and ureases to track the elimination of hydrocarbons has been investigated. Since their activities quickly drop once the rate of biodegradation has decreased, dehydrogenases, catalases, and ureases have been found to be helpful for identifying the start of the bio-degradation process [37]. The ability of the enzymes lipases, dehydrogenases, catalases, and ureases to track the elimination of hydrocarbons has been investigated. Since their activities quickly drop once the rate of biodegradation has decreased, dehydrogenases, catalases, and ureases have been found to be helpful for identifying the start of the bio-degradation process [36].

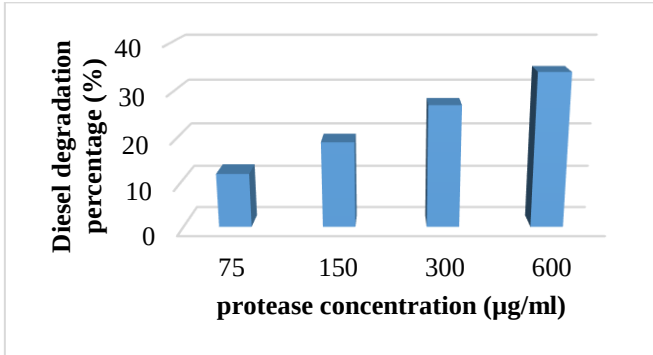

Figure 5. Diesel degradation efficiency at different protease concentrations

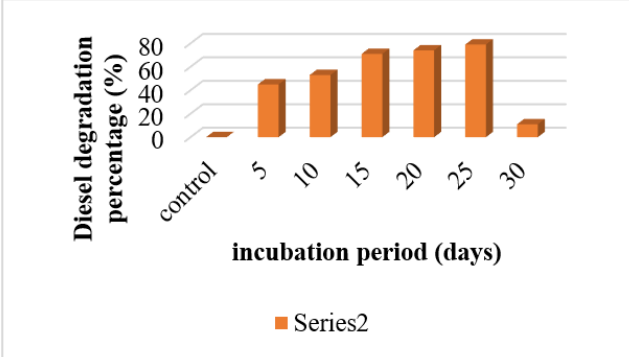

Figure 6. Diesel degradation efficiency at different incubation periods

4. CONCLUSIONS

This study investigated the potential of a purified protease from *Pseudomonas aeruginosa* as a bioremediation agent for diesel hydrocarbon-contaminated environments. While *P. aeruginosa*'s extracellular components, including alkaline protease, are known for their role in pathogenicity and contributing to tissue damage during infection, our findings demonstrate a novel application for this enzyme in environmental cleanup**.**

The core findings of this research are:

- Strain W4 protease was successfully purified with a final yield of 32%.
- The molecular weight of the purified protease was

determined to be 50 kDa.

- The purified protease achieved a diesel hydrocarbon degradation rate of 34% within 24 hours at an enzyme concentration of 600 µg/mL.
- Extending the incubation period led to a significant continuous degradation, reaching 79% after 25 days.

Consequently, this research highlights that purified *Pseudomonas aeruginosa* protease is a highly promising and responsible treatment option. Its demonstrated ability to effectively degrade hydrocarbon-diesel fuel positions it as an efficient and appropriate alternative for cleaning up hydrocarbon-contaminated environments through advanced bioremediation strategies.

ACKNOWLEDGMENT

The authors wish to express their sincere thanks to the Mustansiriyah University / College of Science /Department of Biology for supporting this research.

REFERENCES

[1] Qin, S., Xiao, W., Zhou, C., Pu, Q., Deng, X., Lan, L., Liang, L., Song, X., Wu, M. (2022) *Pseudomonas aeruginosa*: Pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduction and Targeted Therapy, 7: 199. https://doi.org/10.1038/s41392-022-01056-1

[2] Muslim, S.N., Ali, A.N.M, Auda, I.G. (2021). Anti-biofilm and anti-virulence effects of silica oxide nanoparticle–conjugation of lectin purified from *Pseudomonas aeruginosa*. IET Nanobiotechnology, 15(3): 318-328. https://doi.org/10.1049/nbt2.12022

[3] Reynolds, D., Kollef, M. (2021). The epidemiology and pathogenesis and treatment of *Pseudomonas aeruginosa* Infections: An update. Drugs, 81(18): 2117-2131. https://doi.org/10.1007/s40265-021-01635-6

[4] Tang, A., Caballero, A.R., Marquart, M.E., Bierdeman, M.A., O'Callaghan, R.J. (2018). Mechanism of *Pseudomonas aeruginosa* SMALL Protease (PASP), a corneal virulence factor. Investigative Ophthalmology & Visual Science, 59(15): 5993-6002. https://doi.org/10.1167/iovs.18-25834

[5] Muslim, S.N., Muslem, W.H., Alwan, B.H., Rasool, K.H. (2024). Evaluation of L-Asparaginase purified from *Pseudomonas Fluorescens* as inhibitor for biofilm producers in dental decays. Clinical Laboratory, 70(8): 1457-1466. https://doi.org/10.7754/clin.lab.2024.240112

[6] Ołdak, E., Trafny, E.A. (2005). Secretion of proteases by *Pseudomonas aeruginosa* biofilms exposed to ciprofloxacin. Antimicrobial Agents and Chemotherapy, 49(8): 3281-3288. https://doi.org/10.1128/AAC.49.8.3281-3288.2005

[7] Muslim, S.N., Kareem, S.M. (2019). Purification of levanase from Enterobacter aerogenes and using it as a prebiotic and antibacterial agent. International Journal of Drug Delivery Technology, 39(3): 390-395.

[8] Hamza, T.A. (2017). Bacterial protease enzyme: Safe and good alternative for industrial and commercial use. International Journal of Chemical and Biomolecular Science, 3(1): 1-10.

[9] Das, G., Prasad, M. (2010). Isolation, purification & mass production of protease enzyme from *Bacillus subtilis*. International Research Journals of Microbiology, 1(2): 26-31.

[10] Murphy, S.M., Bautista, M.A., Cramm, M.A., Hubert, C.R. (2021). Diesel and crude oil biodegradation by cold-adapted microbial communities in the Labrador Sea. Applied and Environmental Microbiology, 87(20): e00800-21. https://doi.org/10.1128/AEM.00800-21

[11] Nanekar, R.D., Kokitkar, S.S. (2024) Enzyme profiling of petroleum hydrocarbon-degrading bacteria isolated from oil contaminated soils. Indian Journal of Science and Technology, 17(23): 2412-2420. https://doi.org/10.17485/IJST/v17i23.642

[12] Jyothi, N., UMAMAHESWARA, R.V. (2009). Protease and urease production during utilization of diesel by fluorescent Pseudomonas species isolated from local soil. Iranian Journal of Microbiology, 1(3): 23-30.

[13] Alaidaroos, B.A. (2023). Advancing eco-sustainable bioremediation for hydrocarbon contaminants: Challenges and solutions. Processes, 11(10): 3036. https://doi.org/10.3390/pr11103036

[14] Mori, J.F., Kanaly, R.A. (2020). Multispecies diesel fuel biodegradation and niche formation are ignited by pioneer hydrocarbon-utilizing proteobacteria in a soil bacterial consortium. Applied and Environmental Microbiology, 87(1): e02268-20. https://doi.org/10.1128/AEM.02268-20

[15] Baron, E.J. (2001). Rapid identification of bacteria and yeast: Summary of a National Committee for Clinical Laboratory Standards proposed guideline. Clinical Infectious Diseases, 33(2): 220-225. https://doi.org/10.1086/321816

[16] Bergey, D.H. (1994). Bergey's Manual of Determinative Bacteriology. Lippincott Williams & Wilkins.

[17] Shad, A.A., Ahmad, T., Iqbal, M.F., Asad, M.J., Nazir, S., Mahmood, R.T., Wajeeha, A.W. (2024). Production, partial purification and characterization of protease through response surface methodology by Bacillus subtilis K-5. Brazilian Archives of Biology and Technology, 67: e24210355. https://doi.org/10.1590/1678-4324-2024210355

[18] Masi, C., Gemechu, G., Tafesse, M. (2021). Isolation, screening, characterization, and identification of alkaline protease-producing bacteria from leather industry effluent. Annals of Microbiology, 71(1): 24. https://doi.org/10.1186/s13213-021-01631-x

[19] Chandrasekaran, S., Kumaresan, S.S.P., Manavalan, M. (2015). Production and optimization of protease by filamentous fungus isolated from paddy soil in Thiruvarur District Tamilnadu. Journal of Applied Biology and Biotechnology, 3(6): 66-69. https://doi.org/10.7324/JABB.2015.3610

[20] Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2): 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

[21] Ullah, N., Rehman, M.U., Sarwar, A., Nadeem, M., et al. (2022). Purification, characterization, and application of alkaline protease enzyme from a locally isolated Bacillus

cereus strain. Fermentation, 8(11): 628. https://doi.org/10.3390/fermentation8110628
[22] Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685. https://doi.org/10.1038/227680a0
[23] Abdulhussein, H.M., Muslim, S.N., Muslem, W.H. (2025). Bioremediation of petroleum hydrocarbon contaminated soil by xylanase enzyme. Advancements in Life Sciences, 12(1): 255-259. https://doi.org/10.62940/als.v12i1.3125
[24] Ray, C.G., Ryan, K.J. (2014). Sherris Medical Microbiology, Fifth Edition. New York, NY, USA: McGraw-Hill Education/Medical. https://codata.cs.colorado.edu/v10v48hy9cs6/13-gerda-bergstrom-1/s-0071604022.pdf.
[25] Singh, R.K., Sharma, S.R., Singh, A.K., Farooq, U., Singh, S., Sharma, V., Ahamad, I. (2022). Occurrence of *Pseudomonas aeruginosa* isolated from surgical site infections and wound infections and their antimicrobial susceptibility profile. IP International Journal of Medical Microbiology and Tropical Diseases, 8(2): 145-148. https://doi.org/10.18231/j.ijmmtd.2022.030
[26] Vermelho, A.B., Meirelles, M.N.L., Lopes, A., Petinate, S.D.G., Chaia, A.A., Branquinha, M.H. (1996). Detection of extracellular proteases from microorganisms on agar plates. Memorias do Instituto Oswaldo Cruz, 91: 755-760. https://doi.org/10.1590/s0074-02761996000600020
[27] Sun, B., Zou, K., Zhao, Y., Tang, Y., Zhang, F., Chen, W., Tang, X., Chang, C., Zheng, Y. (2023). The fermentation optimization for alkaline protease production by Bacillus subtilis BS-QR-052. Frontiers in Microbiology, 14: 1301065. https://doi.org/10.3389/fmicb.2023.1301065
[28] Yagci, A., Tuc, Y., Soyletir, G. (2002). Elastase and alkaline protease production by Pseudomonas aeruginosa strains: comparison of two procedures. The New Microbiologica, 25(2): 223-229.
[29] Jensen, S.E., Fecycz, I.T., Campbell, J.N. (1980). Nutritional factors controlling exocellular protease production by Pseudomonas aeruginosa. Journal of Bacteriology, 144: 844-847. https://doi.org/10.1128/jb.144.2.844-847.1980
[30] Kessler, E., Kennah, H.E., Brown, S.I. (1977). Pseudomonas protease. Purification, partial characterization, and its effect on collagen, proteoglycan, and rabbit corneas. Investigative Ophthalmology & Visual Science, 16(6): 488-497.
[31] Tahoun, U.M., Hamza, W.T. (2022). *Bacillus subtilis* and *Pseudomonas aeruginosa* as potent protease enzyme producers isolated from the aquatic environment. Egyptian Journal of Aquatic Biology & Fisheries, 26(4): 197-213. https://doi.org/10.21608/ejabf.2022.249552
[32] Karadzic, I., Masui, A., Fujiwara, N. (2004). Purification and characterization of a protease from *Pseudomonas aeruginosa* grown in cutting oil. Journal of Bioscience and Bioengineering, 98(3): 145-152. https://doi.org/10.1016/S1389-1723(04)00258-0
[33] Oh, J., Li, X.H., Kim, S.K. (2017). Post-secretional activation of Protease IV by quorum sensing in *Pseudomonas aeruginosa*. Scientific Reports, 7: 4416. https://doi.org/10.1038/s41598-017-03733-6
[34] Trejos-Delgado, C., Cadavid-Restrepo, G.E., Hormaza-Anaguano, A., Agudelo, E.A., Barrios-Ziolo, L. and Loaiza-Usuga, J.C. (2020). Oil bioremediation in a tropical contaminated soil using a reactor. Anais da Academia Brasileira de Ciências, 92(2): 1-18. https://doi.org/10.1590/0001-3765202020181396
[35] Kanwal, M., Ullah, H., Gulzar, A., Sadiq, T. Gul, Z., Ullah, M. (2022). Biodegradation of petroleum hydrocarbons and the factors effecting rate of biodegradation. American Journal of Biomedical Science and Research, 16(1): 6-15. https://doi.org/10.34297/AJBSR.2022.16.002182
[36] Vetrova, A.A., Nechaeva, I.A., Ignatova, A.A., Puntus, I.F., Arinbasarov, M.U., Filonov, A.E. (2007). Effect of catabolic plasmids on physiological parameters and efficiency of oil destruction by *Pseudomonas* bacteria. Mikrobiologiia, 76: 310-316. https://doi.org/10.1134/S0026261707030071
[37] Izmalkova, T.Y., Sazanova, O.I., Sokolov, S.L., Kosheleva, I.A., Boronin, A.M. (2005). The p-7 incompatibility group plasmids responsible for biodegradation of naphthalene and salicylate in fluorescent pseudomonads. Microbiology, 74: 290-295. https://doi.org/10.1007/s11021-005-0065-0