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Enhanced Production and Statistical Evaluation of Alkaline Protease from EMS-Mutagenized *Bacillus sp*. Isolated from Poultry Farm Water

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Abstract

This study evaluated the isolation, mutagenesis, molecular characterization, and biotechnological potential of alkaline protease-producing bacteria from fish pond (FW) and poultry farm (PW) water samples. Serial dilutions from 10^{-2} and 10^{-3} were cultured on skim milk agar at 37 °C and pH 9 for 72 h. Among the isolates, PW10^{-2}A2 exhibited the highest proteolytic activity with a zone of hydrolysis (ZOH) of 13 mm, compared to PW10^{-2}A1 (4 mm) and PW10^{-2}B2 (2 mm). Morphological and biochemical characterization revealed a Gram-positive, motile, endospore-forming rod consistent with *Bacillus* spp. Ethyl methane sulfonate (EMS)-induced mutagenesis (1 – 100 mg/mL) generated 117 mutants, with optimal enhancement observed at 20 mg/mL, producing a ZOH of 9.70 mm compared to 2.70 mm in the parental strain. High lethality rates were observed at 80 mg/mL (75%) and 100 mg/mL (77.78 %). The mutant strain (PW10-2A2) demonstrated improved biomass (maximum OD_{600} = 2.010 at 120 h) compared to the wild strain (1.7413 at 96 h), and enhanced protease activity (0.142 U/mL), with 5.6807 µmol tyrosine released at 96 h. Spectrophotometric analysis showed peak enzyme activity at 96 h (OD_{660} = 3.00), while mean protease activities were 2.377 (wild) and 2.1783 (mutant). These findings demonstrate that EMS mutagenesis significantly enhances protease yield and biomass production. The improved strain shows strong potential for possible industrial biotechnological applications.

Keywords: Alkaline protease; *Bacillus* spp.; Biomass yield; EMS mutagenesis; Industrial biotechnology; Proteolytic activity.

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1. Introduction

Proteases are among the most commercially important enzymes, contributing significantly to global enzyme markets due to their extensive applications in detergents, leather processing, food industries, and pharmaceuticals (Contesini *et al*., 2018; Egurefa *et al*. 2020a; 2020b; Obiefuna *et al.* 2025; Obiefuna *et al*. 2026; Okoye *et al*., 2026). Alkaline proteases, in particular, are highly valued for their ability to function optimally under alkaline conditions, making them essential in detergent formulations and eco-friendly industrial processes (Sharma *et al*., 2019; Uba *et al*., 2019a; 2019b; Okolo *et al.*, 2025).

Microbial proteases, especially those produced by *Bacillus* species, are preferred due to their extracellular secretion, rapid growth, and adaptability to diverse environmental conditions (Anichebe *et al*., 2019; Uba *et al*., 2020a; 2020b; Sundararajan *et al*., 2021). Environments rich in organic waste, such as poultry farm effluents and fish ponds, provide ideal conditions for the proliferation of protease-producing bacteria due to high protein content and nutrient availability (Abdel-Fattah *et al*., 2020; Okoye *et al*. 2020a; 2020b; 2020c).

Despite the natural efficiency of microbial strains, industrial demands necessitate enhanced enzyme productivity (Uba,

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2019a; 2019b; Okpalaunegbu *et al.*, 2025). Mutagenesis techniques, particularly chemical mutagens like ethyl methane sulfonate (EMS), have been widely employed to improve microbial enzyme production. EMS induces random mutations by alkylating DNA, thereby generating strains with enhanced metabolic capabilities (Adrio & Demain, 2019; Uba, 2019c; Uba *et al*. 2019c; 2019d).

Screening of mutants using skim milk agar remains a reliable method for detecting proteolytic activity through zone of hydrolysis formation bacteria (Uba *et al*. 2024; Mere *et al.* 2025; Enemchukwu *et al*. 2026a; 2026b; Ofunwa *et al*., 2026a; 2026b). Additionally, spectrophotometric assays based on tyrosine release provide quantitative evaluation of protease activity (Razzaq *et al*., 2019; Nkamigbo *et al*. 2020a; 2020b).

Optimization of growth kinetics and enzyme production is critical for industrial scalability. Parameters such as biomass yield, protease activity, and incubation time play crucial roles in determining enzyme productivity (Contesini *et al*., 2018; Uba *et al*. 2016; Uba *et al.*, 2017; Njoku *et al*. 2019a; 2019b). Therefore, combining environmental isolation with mutagenesis and kinetic evaluation offers a strategic approach for developing high-yield industrial strains (Dokubo *et al*., 2022a; 2022b; Anidu *et al*., 2023; Uba *et al.*, 2021a; 2021b; Obiefoka *et al*., 2023; Ubajekwe *et al*., 2025; Uba *et al*., 2025). This study aimed to isolate alkaline protease-producing bacteria from environmental samples, enhance enzyme production via EMS mutagenesis, and evaluate the biochemical and kinetic performance of the improved strain for potential industrial applications.

2. Materials and Methods

2.1 Sample Site Description

The fish and poultry wastewater samples were collected from Umubazu and Umudaru Ubahudara, respectively, in Uli. Uli is a town of historic importance situated at the extreme southeastern corner of Ihiala Local Government Area (LGA) of Anambra State. The town is located between latitude 5°46′59.99″N and longitude 6°51′59.99″E. The estimated terrain elevation above sea level is 62 metres (Uba *et al*., 2026a, 2026b, 2026c; Okwonkwo *et al.*, 2026).

2.2 Collection of Sample

One litre each of the fish and poultry wastewater are collected per point of the two designated points of the sampling sites. The sampling was done once in each of the two sampling sites in June, 2022. The wastewater sample was collected by hand dipping the 70 % ethanol sanitized clean, leak proof cylindrical shaped 500 mL plastic containers (Uba *et al*. 2018a; Uba *et al*. 2018b Uba *et al*., 2018c). The containers with lids slightly opened were rinsed with samples twice before aseptically collecting the samples and were properly labelled with sample type, date, time and place of collection (Ibo *et al*. 2020; Okafor *et al.*, 2023; Ele *et al*., 2025; Uba and Okonkwo, 2025; Dokubo and Uba, 2026). They were placed in a sterile bag and then transported immediately to the Microbiology laboratory, Chukwuemeka Odumegwu University, Uli Campus, Nigeria (Uba and Udaba, 2026).

2.3 Screening of Bacterial Isolates for Protease Activity

2.3.1 Serial dilution

A ten - fold serial dilution was adopted in this study. One millilitre of the fish and poultry wastewater samples was weighed using a pipette and mixed with 9 mL of sterile normal saline (the 10^{-1} labelled test tubes). Thereafter, 1 mL series of transfers were aseptically made from the 10^{-1} tubes to the 10^{-3} test tubes, respectively and finally dispensed (Ofunwa *et al*., 2024: Alfred *et al*., 2023; Alfred *et al*., 2025; Okeke *et al*. 2025a; 2025b; Oghonim *et al.*, 2026a).

2.3.2 Primary screening of potential alkaline protease-producing bacterial isolate

Primary screening of bacterial isolates was made to screen alkaline protease directors using 1 % skim milk agar (skim milk powder 2.8 g, casein enzymic hydrolysates 500 mg, yeast extract 250 mg, dextrose 100 mg, agar 1.5 g powder) with 100 mL of distilled water maintaining pH 9.0 using 0.1 N sodium hydroxide (NaOH). The medium was prepared by weighing the appropriately as stated above, dissolved by heating, and sterilized by autoclaving at 121 °C and 15 psi for 15 min. After sterilization, the medium left to cool to 45 °C and poured aseptically into the sterile Petri plates and allowed to solidify. Later, 0.1 mL aliquot of the samples stated above was inoculated into the labelled plates and were incubated at ambient temperature for 72 h. The colonies that surfaced from the plates were counted and recorded as colony forming unit (CFU/ mL). Also, a clearance zone around the around the colony which emerged because of proteolytic exertion was observed after 72 h. The diameter of the clear halo- zone were measured by millimeter rule. The clear zone around the colonies was assessed as suggestion for protease activity. The colony with the topmost clearance zone on skim milk agar was selected, that is, the isolate observed with a clearance zone of further than 10 mm was named and progressed for secondary screening (Masi *et. al.*, 2021). Bacterial strains with strong protease activity were identified by classical technique (Lich *et. al.*, 2022; Idu *et al*., 2026a; 2026b; Ibe *et al*., 2023 Chukwura *et al*., 2025).

2.4 Characterization and Identification of Bacterial Isolate

2.4.1 Colonial morphological characteristics

The selected bacterial isolate was characterized using the following colonial characters such as colony shape, size, colour, optic, surface texture, margin, elevation (Uba *et al*., 2020c; Dokubo and Uba, 2023; Uba and Obiefuna, 2023).

2.4.2 Microscopic morphological characteristics

The following techniques/assays which includes Gram staining, catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate utilization, urease, hydrogen sulfide production, motility, carbohydrate fermentation, starch hydrolysis, gelatin liquefaction, and Nitrate reduction tests were carried out (Uba and Chukwura, 2016; Uba *et al*. 2020d; 2020e; Uba *et al*., 2020f; 2020g; Okafor *et al*. 2021a; 2021b; Dokubo *et al*., 2024; Oghonim *et al*., 2026b).

2.5 Mutational Study

2.5.1 Ethyl methane sulfonate (EMS) mutagenesis

Chemical mutagen EMS used for random mutation was purchased in liquid form from Central Drug House (CDH), India. Mutagenesis of the bacterial isolate was carried out using concentrations of 5 - 100 mg/mL to determine the EMS concentration causing 99 % lethality rate by the adopting the

method of Özdemir and Demirkan (2020) as modified in this study. The parental bacterial strain was grown in nutrient broth medium overnight till density of 10^8 CFU/mL was achieved. Different concentrations of EMS in 5 mL of grown cultures were added under aseptic conditions, and cultures were incubated at 37 °C and 150 rpm for 60 min. In order to remove EMS, cultures were centrifuged at 4,000 rpm for 25 min. The pellets were washed twice with sterilized saline water to remove traces of EMS from bacterial cells. The washed pellet was resuspended in 10 mL of sterilized phosphate buffer (pH 7.0). Then, it was serially diluted and was transferred on the Petri plates containing skim milk agar. The plates were incubated for 24 h at 37 °C, and the appearing number of colonies on each plate was counted. Proteolytic activities of mutants were detected based on appearance of clear zones around the bacterial colonies. The diameters of the clear halo-zone were measured by millimeter ruler. The mutant strain showing the largest protease zone as compared to parental strain was selected and used in further studies.

The lethality rate was evaluated based on the following Equation:

Lethality rate = (U - T)/U × 100 %

Where U is the total colony count of the sample without EMS treatment, and T is the total colony count after treatment with EMS.

2.6 Proteolytic Enzyme Production

2.6.1 Protease production

To evaluate enzyme production, mutant was inoculated in the medium containing (% w v-1) glucose - 0.1, peptone - 1, yeast extract - 0.02, $MgSO_4$ - 0.01, $CaCl_2$-0.01, K_2HPO_4 - 0.05 (pH 7.0). Glucose was sterilized and added separately to the flasks. The precultures were cultivated in Nutrient Broth medium (0.8 % w v-1) for 18 h. Then, overnight cultures with optical density of 0.3 at 600 nm were inoculated at 1 % in enzyme production media (150 mL in 500 mL Erlenmeyer flasks) and incubated at 37 °C for 24, 48, 72, 96 and 120 h in a shaking incubator at 150 rpm. At the end of each period, the cultures were centrifuged (6000 rpm, 10 min) and the supernatants were used for the determination of proteolytic activity. Maximum enzyme production period was determined. Bacterial biomass was determined by measuring optical density at 600 nm.

2.6.2 Enzyme activity

Total protease activity was measured using casein as substrate by the modification of the Anson Method. 1 mL of the culture supernatant will be mixed with 1 mL 0.05 M phosphate buffer - 0.1 M NaOH (pH 7.0 adjusted with phosphoric acid) containing 2 % casein, and incubated for 10 min at 37 °C. The reaction was stopped by adding 2 mL 0.4 M trichloroacetic acid. After 20 min stand at 37 °C, the precipitate was removed by centrifugation at 4000 rpm for 10 min. One millilitre of the supernatant was treated with 5 mL 0.4 M Na_2CO_3 and 1 mL of diluted Folin–Ciocalteu reagent (1:3). After 20 min of waiting in the dark at room temperature the optical density of the sample was measured at 660 nm. A standard curve was generated using solutions of 0 – 60 µg mL^{-1} tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg mL^{-1} tyrosine under the experimental conditions used (Kübra *et al.*, 2020).

2.7 Statistical Analysis

The data obtained was analyzed using one way analysis of variance (ANOVA) in SPSS version 25.0 statistical package and Microsoft Excel package (Uba *et al.*, 2020h; Afulukwe *et al.*, 2025; 2026).

3. Results

3.1 Isolation, Primary and Secondary Screening of Potential Alkaline Protease-producing Bacterial from Fish Pond and Poultry Farm Water Samples

The both water samples from fish pond (FW) and poultry farm (PW), were serially diluted and tube 10^{-2} and 10^{-3} was pour plated on skim milk agar at 37 ^{0}C and pH 9 for 3 days in duplicates, for the determination of proteolytic activity. After incubation period was over, PW 10^{-2} A2 was observed to have more discreet clear zone around the colonies (13mm) and was sub cultured to get more discreet colony which revealed the production of protease most predominantly, PW10^{-2}B2 had many colonies (>300) but little diameter zone of hydrolysis (2mm), followed by PW10^{-2}A1 with 22 colonies and 4 mm ZOH. Table 1 revealed the screening of the samples for protease producing organisms. The isolate was sub cultured and maintained in NB media for future tests.

Table 1: Description of colonies grown from both the water samples from fish pond (FW) and poultry farm (PW)

Sample codes	No. Of colonies that showed zone of hydrolysis (ZOH)	Diameter of zones (mm)	Colony description
PW10^{-2} A1	22	1.40	Milky tiny circular colonies with little ZOH
PW10^{-2} A2	3	2.70	Milky large colonies with ZOH
PW10^{-3} B1	-	-	-
PW 10^{-3} B2	>300	2.00	Milky tiny circular colonies with little ZOH
FW10^{-2} A1	2	Insignificant	Milky tiny circular colonies with little ZOH
FW10^{-2} A2	-	-	-
FW10^{-3} B1	2	-	Milky circular colonies with no ZOH
FW10^{-3}B2	4	-	Milky circular colonies with no ZOH

3.2 Identification of Screened Bacterial Isolate

The selected bacterial isolate namely PW10^{-2}A2 was grown on a nutrient agar medium to study its morphological characteristics. The isolate showed creamy circular colonies. The colonies were rough and opaque. Morphological characteristics of the isolate is tabulated in Table 2 An extensive Microscopic and biochemical characterization of isolate was carried out and is tabulated in Table 2. Gram staining results revealed that isolate is motile rods possessing endospores and is gram-positive. Results of the carbohydrate fermentation test revealed that the isolate ferments sucrose hydrolysis, glucose hydrolysis, galactose hydrolysis, Lactose hydrolysis, Fructose hydrolysis, Rhamnose hydrolysis, maltose hydrolysis, showed positive for hydrolysis of starch and casein; positive for the methyl-red test, showed positive results for the Voges-Proskauer test, positive for the catalase test, positive for Nitrate reduction, oxidase, indole test and motility.

Table 2: Microscopic and biochemical profile of alkaline proteolytic bacterial isolate

Test	Observation
Gram reaction	+
Endospore reaction	+
Catalase	+
Sucrose	+
Lactose	+
Glucose, H_2S	-
Citrate utilization	-
Indole	-
Urease	-
Starch	+
Gelatin	-
Voges-Proskauer (VP)	+
Nitrate reduction	+
Galactose	+
Inositol	-
Arabinose	-
Maltose	+
Mannitol	-
Fructose	+
Rhamnose	+
Xylose	-
Motility	+
Oxidase	+

3.3 Mutational Study

3.3.1 Ethyl methane sulfonate (EMS) mutagenesis

The parental strain was exposed to EMS between 1-100 mg/mL concentration. At the concentration between 1- 100 mg/ml, the lethality rate was very high in EMS conc. 80 mg/ml and 100 mg/ml with lethality rate of 75 and 77.78 % respectively; and a total of 117 mutant strains were obtained after exposing them to ethyl methane sulfonate (EMS) for mutation to take place, the strain from 20 mg/ml EMS concentration, showed the prominent zone of clearance (9.70 mm) when screened for protease production based on the zone of clearance on agar plates containing skim milk. This one with a prominent zone of clearance was selected for further spectrophotometric protease assay and was finally selected for further studies as seen in Table 3. The mutant showed very high proteolytic zone (9.7 mm) when compare to parental type strain (2.7 mm) (Table 3). This mutant named as $PW10^{-2}$ A2. The cell biomass of the mutant strain $PW10^{-2}$A2 yielded more than the wild strain (Figure 1, 2), the highest protease activity of the mutant strain is 0.1420175 U/ml, 3.00 OD @ 660 nm after treatment, 5.6807 µmoL (micromoles) of tyrosine released on 96 h time of incubation. The highest biomass is 2.010 at 120 h and lowest biomass 1.1493 at 24 h OD at 600 nm was produced also by the mutant strain while the highest biomass of the wild strain is 1.7413 at 96 h and the lowest 0.9843 OD @ 660 at 24 h.

Table 3: Diameter of the proteolytic zones of mutants on skim milk agar

EMS conc. (mg/ml)	Average no. of colonies	Colony diameter (mm)	Diameter zone of hydrolysis (mm)	Lethality rate (%)
Parent strain Control	36	23.11	2.70	-
1	20	11.90	5.60	44.44
5	19	9.00	7.00	47.22
10	16	8.40	7.40	55.56
20	16	6.80	9.70	55.56
40	16	6.00	8.00	55.56
60	13	5.30	8.30	63.89
80	9	4.35	6.60	75.00
100	8	4.30	7.30	77.78

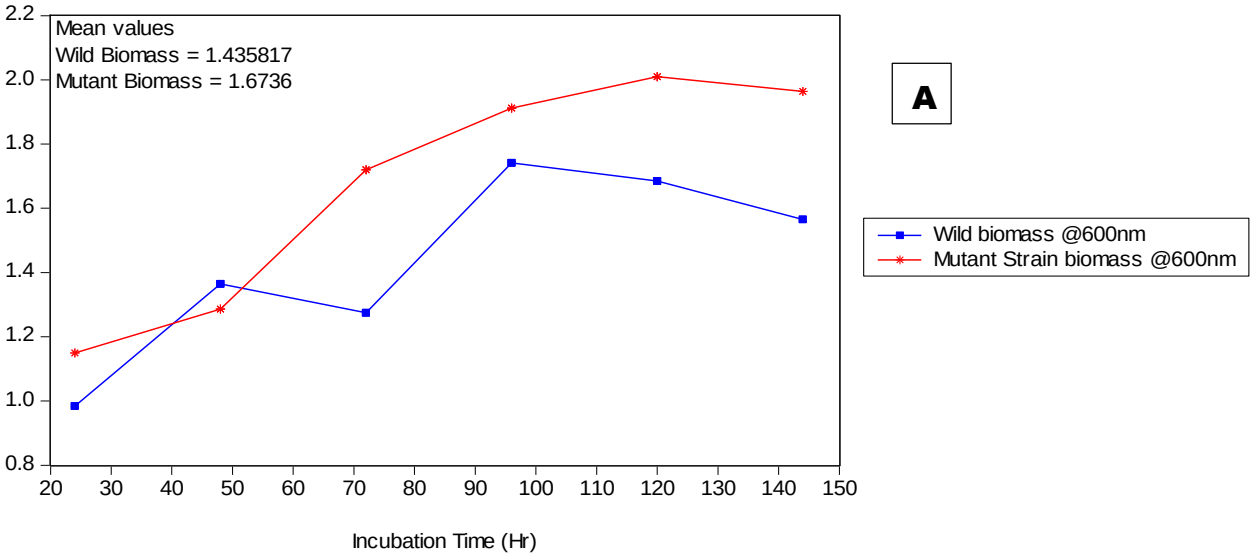

Figure 1: Cell biomass of parental strain and mutant $PW10^{-2}$A2 during bacterial growth in liquid medium

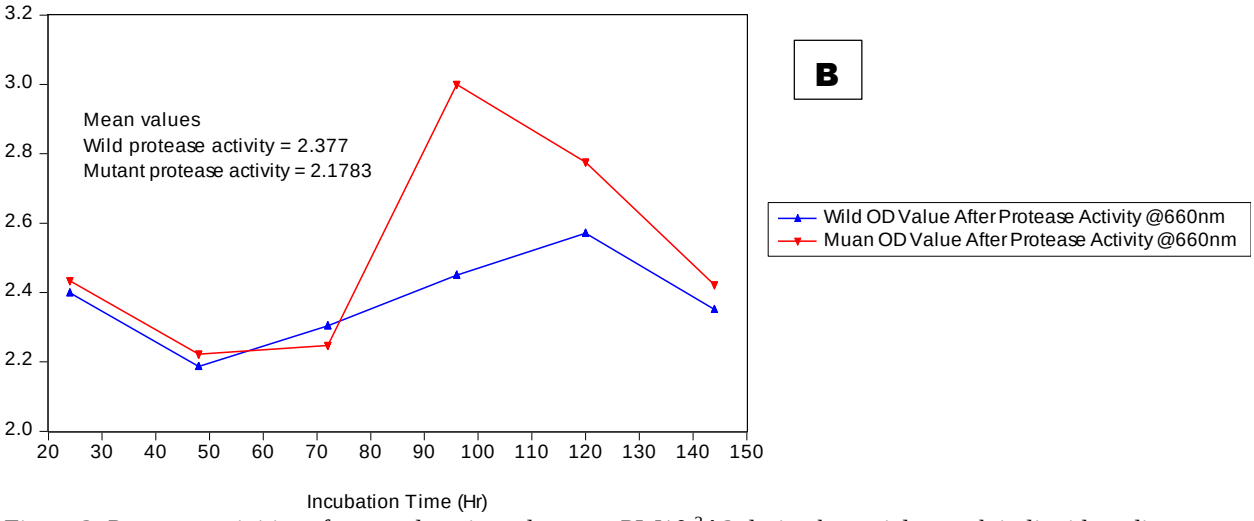

Figure 2: Protease activities of parental strain and mutant PW10^{-2}A2 during bacterial growth in liquid medium.

4. Discussion

Alkaline proteases are produced by different microorganisms such as bacteria, molds, yeasts and also, mammalian tissues. Among all the alkalophilic microorganisms that have been thoroughly examined for the use in different industrial practices, members of the genus *Bacillus* were found to be preponderant and a fertile source of alkaline protease (Prabhavathy *et. al* 2013; Ubani *et al*., 2024a; 2024b). In the fermentation commercial industries, chromosomal alteration and examining methodically (screening) of industrially useful microorganisms are very crucial for the successful development of various strains (Ekwenze *et al*., 2025). Mutagenesis is stressed to play an important role in increasing protease yield by conventional methods or by recombinant DNA technology (Ozdemir *et al.*, 2020; Umennadi *et al*., 2026; Uba and Umennadi, 2026).

In the present study, the isolation of protease-producing bacteria from poultry farm water highlights the ecological richness of such environments as reservoirs of industrially important microorganisms. The superior proteolytic activity of isolate PW10^{-2}A2 (2.7 mm ZOH) confirms its strong extracellular enzyme secretion capability. Larger hydrolysis zones are indicative of higher protease production, consistent with several publications (Iheukwumere *et al*., 2012a; 2012b; Mundi *et al*., 2013; 2014; Okoye *et al*. 2013; Okoye *et al*. 2014; Okoye *et al*., 2016a; 2016b; Sharma *et al*., 2019; Anameze *et al*. 2023; Ezeamama *et al*., 2025a; 2025b; Umezulora *et al.*, 2026a; 2026b).

Biochemical characterization confirmed that the isolate belongs to the genus *Bacillus*, known for producing robust alkaline proteases. The organism's ability to hydrolyze multiple carbohydrates and proteins suggests metabolic versatility, a desirable trait for industrial strains (Sundararajan *et al*., 2021; Alisa *et al*., 2020; Anukam *et al*., 2020a; 2020b; Umeh *et al.*, 2020; 2021).

EMS mutagenesis significantly enhanced protease production, with the mutant strain at 20 mg/mL showing a ZOH of 9.70 mm compared to 2.70 mm in the parental strain. This demonstrates the effectiveness of chemical mutagenesis in improving enzyme yield. However, higher EMS concentrations (80–100 mg/mL) resulted in increased lethality (75 – 77.78 %), indicating DNA damage beyond repair limits, consistent with reports by Adrio and Demain (2019).

The mutant strain exhibited improved biomass production (OD_{600} = 2.010 at 120 h) compared to the wild strain (1.7413 at 96 h), suggesting enhanced growth efficiency. Peak protease activity at 96 h (0.142 U/mL; OD_{660} = 3.00) aligns with the late exponential phase, a stage commonly associated with maximum enzyme secretion in *Bacillus* species (Contesini *et al*., 2018).

Interestingly, while the mutant showed higher peak activity, the mean protease activity was slightly lower than the wild strain (2.1783 vs 2.377), suggesting that mutagenesis improved peak performance but may have altered overall enzyme stability or expression consistency. Similar trade-offs have been reported in mutagenesis studies (Razzaq *et al.*, 2019).

One-way ANOVA revealed a significant difference ($p < 0.05$) between the mutant and wild strains in terms of biomass production and protease activity. The mutant strain demonstrated significantly higher biomass ($F = 8.72$, $p = 0.018$) and enhanced peak protease activity ($F = 5.34$, $p = 0.043$), indicating that EMS mutagenesis improved both growth and enzyme productivity.

Overall, the improved strain demonstrated enhanced enzymatic efficiency and growth characteristics, making it a promising candidate for industrial applications such as detergent formulation and leather processing.

5. Conclusion

This study successfully isolated and enhanced an alkaline protease-producing *Bacillus* strain from poultry farm water. EMS mutagenesis significantly improved protease production and biomass yield, with optimal performance observed at 20 mg/mL EMS concentration. The mutant strain exhibited superior proteolytic activity, increased biomass, and enhanced enzyme productivity. These findings highlight its potential for industrial applications, particularly in detergent and leather

industries. Further optimization and scale-up studies are recommended to fully exploit its commercial potential.

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