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Bioprocess Optimization of Alkaline Protease Production by *Bacillus tropicus*: Effects of Culture Conditions and Nutritional Factors

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Abstract

This study investigated the isolation, characterization, and optimization of alkaline protease production by a bacterial isolate obtained from fish pond (FW) and poultry farm (PW) water samples. Serial dilutions 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 identified the isolate as a Gram-positive, motile, endospore-forming rod with broad carbohydrate utilization and enzymatic capabilities, consistent with *Bacillus tropicus*. The Optimization studies revealed that maximum protease production occurred at 96 h (0.1420 Umol/mL; 5.6807 µmol tyrosine released; biomass 1.912 OD_{600}), while minimum production was observed at 48 h (0.1052 Umol/mL; 4.2075 µmol tyrosine; biomass 1.286 OD_{600}). Among carbon sources, rice bran yielded the highest activity (0.1567 Umol/mL), whereas maltose showed the lowest (0.1085 Umol/mL). Beef extract was the best organic nitrogen source (0.1671 Umol/mL), while ammonium nitrate (NH_4NO_3) was the most effective overall nitrogen source (0.17435 Umol/mL). Optimal enzyme production occurred at pH 9 (0.1425 Umol/mL) and 35 °C (0.0703 Umol/mL). Maximum yield was achieved with 2.5 mL inoculum size (0.1314 Umol/mL), 96 h inoculum age (0.1305 Umol/mL), and agitation speed of 220 rpm (0.0605 Umol/mL). One-way ANOVA demonstrated that incubation time, carbon and nitrogen sources, pH, temperature, inoculum size, inoculum age, and agitation significantly influenced protease production ($p < 0.05$). Maximum enzyme yield was obtained under optimized conditions (96 h, pH 9, 35 °C, rice bran, NH_4NO_3, 2.5 mL inoculum, 220 rpm). Correlation analysis indicated that enzyme production was not strictly growth-associated ($r = 0.42$). The study demonstrates that *Bacillus tropicus* PW10^{-2}A2 is a potent alkaline protease producer with significant industrial potential, particularly for applications in detergent and leather processing industries.

Keywords: Alkaline protease; *Bacillus tropicus*; Enzyme optimization; Fermentation parameters; Industrial biotechnology; Protease kinetics

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1. Introduction

Proteases (EC 3.4.x.x) constitute one of the most important classes of industrial enzymes, accounting for approximately 60% of the global enzyme market due to their extensive applications in detergents, leather processing, food industries, pharmaceuticals, and environmental bioremediation (Contesini *et al*., 2018; Razzaq *et al*., 2019; Egurefa *et al*. 2020a; 2020b; Obiefuna *et al.* 2025; Obiefuna *et al*. 2026; Okoye *et al*., 2026). These enzymes catalyze the hydrolysis of peptide bonds in proteins, converting complex macromolecules into smaller peptides and amino acids. Among the various classes of proteases, alkaline proteases have gained considerable attention because of their optimal activity and stability under alkaline conditions (pH 8 – 11), which makes them particularly suitable for industrial processes such as detergent formulation and leather dehairing (Sharma *et al*., 2019; Uba *et al*., 2019a; 2019b; Okolo *et al.*, 2025).

Microorganisms are the preferred sources of industrial proteases due to their rapid growth, ease of genetic manipulation, and ability to secrete extracellular enzymes in large quantities. Bacterial species, especially those belonging

to the genus *Bacillus*, are widely recognized for their capacity to produce highly stable and efficient alkaline proteases (Anichebe *et al*., 2019; Uba *et al*., 2020a; 2020b; Sundararajan *et al*., 2021). Members of this genus are known for their adaptability to diverse environmental conditions, including extreme pH, temperature, and salinity, making them ideal candidates for industrial enzyme production. In particular, *Bacillus tropicus* has emerged as a promising species with significant enzymatic potential, although it remains relatively underexplored compared to other *Bacillus* species (Okoye *et al*. 2020a; 2020b; 2020c).

Environmental habitats rich in organic matter, such as fish ponds and poultry farm effluents, are excellent sources for isolating protease-producing microorganisms. These environments contain high levels of proteinaceous materials and nitrogenous wastes, which create selective pressure for microorganisms capable of producing extracellular proteases to utilize these substrates (Uba, 2019a; 2019b; Abdel-Fattah *et al*., 2020; Okpalaunegbu *et al.*, 2025). Despite their potential, such ecological niches—especially in developing regions—remain underutilized for biotechnological exploration, highlighting the need for systematic isolation and screening of indigenous microbial strains (Uba, 2019c; Uba *et al*. 2019c; 2019d).

The production of microbial proteases is significantly influenced by cultural and nutritional factors, including incubation time, pH, temperature, carbon and nitrogen sources, inoculum size, and agitation rate. Optimization of these parameters is essential for maximizing enzyme yield and ensuring cost-effective industrial production (Contesini *et al*., 2018). For example, agro-industrial by-products such as rice bran have been widely reported as economical carbon sources that enhance microbial growth and enzyme production, thereby promoting sustainable bioprocessing (Razzaq *et al*., 2019; Uba *et al*. 2024; Mere *et al.* 2025). Similarly, the type and concentration of nitrogen sources play a crucial role in regulating enzyme synthesis, with both organic and inorganic sources affecting metabolic pathways differently (Enemchukwu *et al*. 2026a; 2026b; Ofunwa *et al*., 2026a; 2026b).

In addition to optimization, understanding the relationship between microbial growth (biomass) and enzyme production is critical. In many cases, enzyme synthesis does not directly correlate with biomass accumulation, as protease production is often a secondary metabolic process that occurs during the late exponential or stationary phase of growth (Sharma *et al*., 2019; Nkamigbo *et al*. 2020a; 2020b). Therefore, kinetic studies evaluating parameters such as enzyme activity, biomass yield, and incubation time are essential for identifying optimal production conditions (Uba *et al*. 2016; Uba *et al.*, 2017; Njoku *et al*. 2019a; 2019b).

Furthermore, the increasing demand for eco-friendly and sustainable industrial processes has driven interest in enzymatic alternatives to conventional chemical methods. For instance, alkaline proteases are widely used in detergent formulations for the removal of protein-based stains and in leather processing for dehairing, replacing harsh chemicals such as sodium sulfide that pose environmental and health risks (Sundararajan *et al*., 2021; Uba e*t al.*, 2021a; 2021b; Dokubo *et al*., 2022a; 2022b; Anidu *et al*., 2023). This shift toward green technology underscores the importance of discovering and optimizing new enzyme-producing strains with high efficiency and stability (Obiefoka *et al*., 2023; Ubajekwe *et al*., 2025; Uba *et al*., 2025).

Despite extensive research on microbial proteases, there remains a continuous need to identify novel strains with enhanced production capacity and adaptability to industrial conditions. Therefore, this study aimed to isolate and characterize alkaline protease-producing bacteria from fish pond and poultry farm water samples, identify potent strains based on proteolytic activity, and optimize the physicochemical and nutritional parameters influencing enzyme production.

2. Materials and Methods

2.1 Sample Site Description

The area from which the samples (fish and poultry waste water) were collected from Umubazu and Umudaru Ubahudara, respectively in Uli. Uli is a town of historic importance situated at extreme southeast corner of Ihiala LGA of Anambra state, this town is located between the latitude 5^0 46'59.99"N and longitude $6^0$51'59.99"E. the estimate 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 *et al*. 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 *et al*. 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 Optimization Study for Culture Conditions for Alkaline Protease Production

The bacterial strain with the highest protease was used for determining the effect of different parameters such as pH, temperature in the production of protease by mutant bacteria (Ubani *et al*., 2024a; 2024b; Ubani *et al.,* 2025; Ekwenze *et al*., 2025).

2.5.1 Effect of nutritional factors on protease production

Various carbon sources (0.1 %) such as glycerol, cellulose, sucrose, corn starch, maltose, rice bran, cassava peels, yam peels evaluated for their effect on protease production by replacing glucose in the basal medium. Organic nitrogen sources chosen for the study were beef extract, skim milk, cow dung, plantain peels and corn step liquor by replacing peptone and yeast extract (total 1.02 %); and inorganic nitrogen sources were NH_4NO_3, KNO_3 and $NaNO_3$. These nitrogen sources were used to replace the organic and inorganic source available in the basal medium. Protease activity and biomass were determined for all the samples.

2.5.2 Effect of other physical factors on protease production

Some parameters (temperature, pH, agitation, inoculum size and inoculum age (days)) was studied for its influence in protease production in basal medium. The effect of temperature was performed by incubating the reaction mixtures at different temperatures such as 15, 25, 35, 45 and 55 °C in basal medium with optimal pH of 9.0. The pH in the range of 3, 5, 7, 9, and 11 were examined for their effect on protease production by the mutant strain in basal medium. Effect of agitation condition was carried out at following shaking rate, 0 rpm, random rpm, 80 rpm,150 rpm and 220 rpm. The mutant strain was grown in basal medium until OD 600 of 0.3 and the cultivation was carried out at different inoculum sizes ranging (2.5, 5, 7, 10 and 15 mL). Protease activity and biomass was measured by incubating production medium seeded with different inoculum age (24, 48, 72, 96 and 120 h). Protease activity and biomass was determined for all the samples. Protease and biomass yield were recorded, and compared with basal medium.

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 (13 mm) 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 (2 mm), 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	40	Milky tiny circular colonies with little ZOH
$PW10^{-2}$ A2	3	13	Milky large colonies with ZOH
$PW10^{-3}$ B1	-	-	-
PW 10^{-3} B2	>300	2	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 1. 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 Optimization Profile on Protease Product by *Bacillus tropicus* PW10^{-2}A2

Effect of different incubation time on the production of protease enzyme and to determine for maximum enzyme yield was determined as shown in Figure 1. The maximum enzyme production was observed at 96 h with protease activity of 0.1420175 µmol (5.6807 micromoles of tyrosine released, biomass of 1.912 @ OD 600 nm) and the minimum yield at 48 h with protease activity of 0.1051875 µmol/mL (4.2075 micromoles of tyrosine released, biomass of 1.286 @ OD 600 nm). The protease activity declined down the time after the maximum while the biomass increased and then declined.

Among the different carbon sources tested, rice bran was found with the highest protease activity of 0.1567 µmol/mL (6.268 micromoles of tyrosine released, biomass of 2.205 @ OD 600 nm) and the lowest activity was found to be maltose, with 0.1085475 µmol /mL (4.341 micromoles of tyrosine released, biomass of 2.162 @ OD 600 nm) as shown in Figure 2. Bacterial growth (biomass) was not parallel with enzyme production.

Beef extract was found to be the best organic nitrogen source by *Bacillus tropicus* PW10^{-2}A2 to produce maximum protease enzyme of 0.1671 µmol/mL (6.684 moles of tyrosine released, biomass of 1.947 @ OD 600 nm) and cow dung was found with the lowest yield of 0.135725 µmol/mL (5.429 micromoles of tyrosine released, biomass of 0.991 @ OD 600 nm). NH_4NO_3 was found with the highest yield of 0.17435 µmol/mL (6.974 micromoles of tyrosine released, biomass of 0.949 @ OD 600 nm) and $NaNO_3$ had the lowest yield of 0.09855 µmol/mL (3.942 micromoles of tyrosine released, biomass of 0.764 @ OD 600 nm) among the inorganic nitrogen sources. NH_4NO_3 is the overall best when compared with both the inorganic and organic nitrogen sources used in this study (Figures 3a and 3b).

Bacillus tropicus PW10^{-2}A2 produced the highest protease enzyme at pH 9 (0.142475 µmol/mL, 5.6997 moles of tyrosine released, biomass of 1.942 @ OD 600 nm) and lowest at pH 3 (0.122125 µmol/mL, 4.8854 micromoles of tyrosine released, biomass of 0.009 @ OD 600 nm) (Figures 4a and 4b).

Effect of various cultivation temperatures on the production of protease enzyme was determined as shown in Figures 5a and 5b. The highest protease yield was seen at 35 ^{0}C (0.0703 µmol /mL, 2.812 micromoles of tyrosine released, biomass of 1.954 @ OD 600 nm) and the lowest at 15 ^{0}C (0.04795 µmol/mL, 1.918 micromoles of tyrosine released, biomass of 1.805 @ OD 600 nm). The protease yield by *Bacillus tropicus* PW10^{-2}A2 was seen gradually degreasing down the temperature degrees on cell biomass (Figure 5b) with a slight increase after 35 ^{0}C for protease activity, respectively.

The maximum alkaline protease yield was observed with 2.5 mL (0.131425 µmol/mL, 5.257 micromoles of tyrosine released, biomass of 2.010 @ OD 600 nm) inoculum size and the lowest was observed with 10 mL (0.07025 µmol/mL, 2.8100 micromoles of tyrosine released, biomass of 2.179 @ OD 600 nm) (Figures 6a and 6b).

As seen in Figure 7, 96 h inoculum age was observed to have the highest alkaline protease yield of (0.130525 µmol/mL, 5.221 micromoles of tyrosine released, biomass of 2.004 @ OD 600 nm) and lowest at 72 h (0.062925 µmol/mL, 2.517 micromoles of tyrosine released, biomass of 2.068 @ OD 600 nm).

Effect of different agitation rpm on the production of alkaline protease of *Bacillus tropicus* PW10^{-2}A2 was determined as shown in Figure 8. The lowest alkaline protease yield was observed at 80 rpm (0.0569 micromole/mL, 2.276 micromoles of tyrosine released, biomass of 1.559 @ OD 600 nm) and the highest was observed at 220 rpm (0.0604975 µmol/mL, 2.4199 micromoles of tyrosine released, biomass of 2.167 @ OD 600 nm).

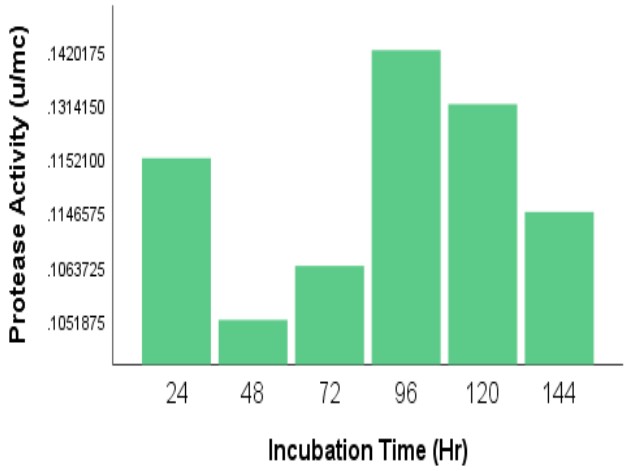

Figure 1: Effect of incubation period on growth and production of alkaline protease by *Bacillus tropicus* PW10^{2}A2

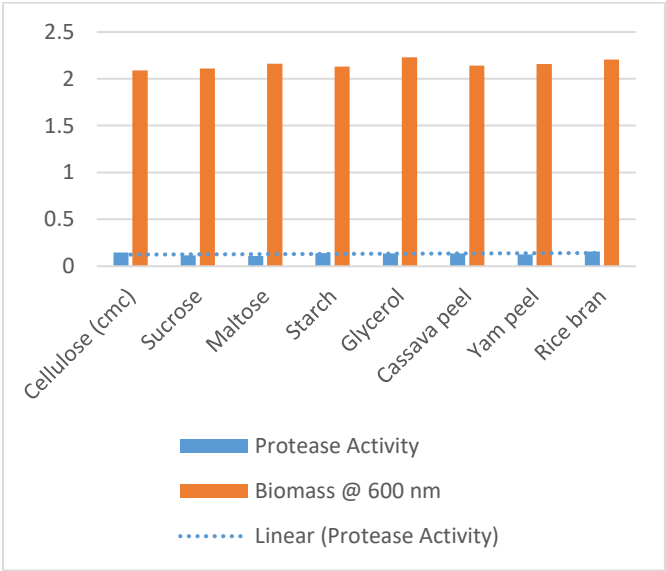

Figure 2: Effect of carbon sources on growth and production of alkaline protease by *Bacillus tropicus* PW10^{2}A2.

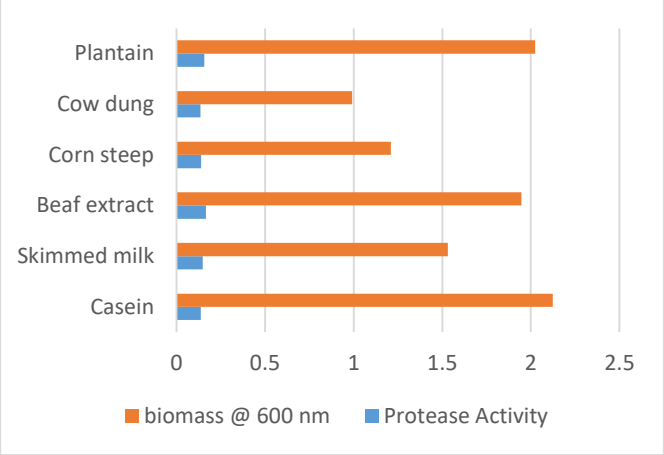

Figure 3a: Effect of organic Nitrogen sources on growth and production of alkaline protease by *Bacillus tropicus* PW10^{-2}A2

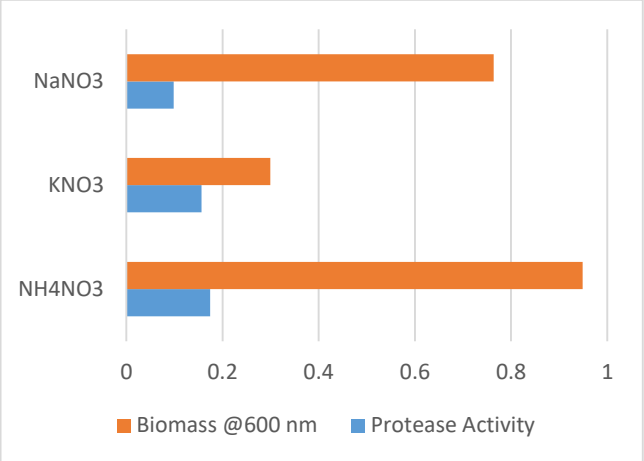

Figure 3b: Effect of inorganic nitrogen sources on growth and production of alkaline protease by *Bacillus tropicus* PW10^{-2}A2

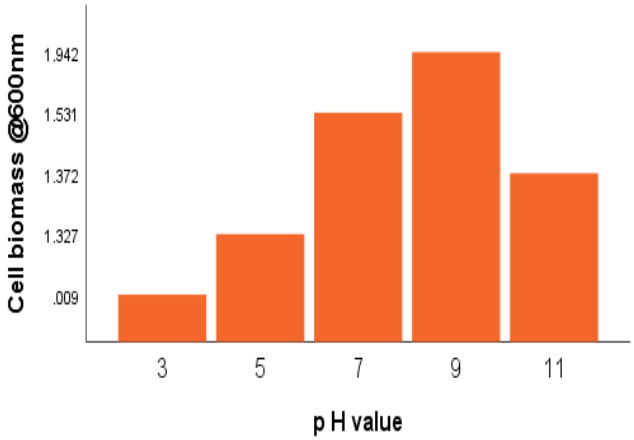

Figure 4a**:** Effect of pH on growth on cell biomass on *Bacillus tropicus* PW10^{-2}A2

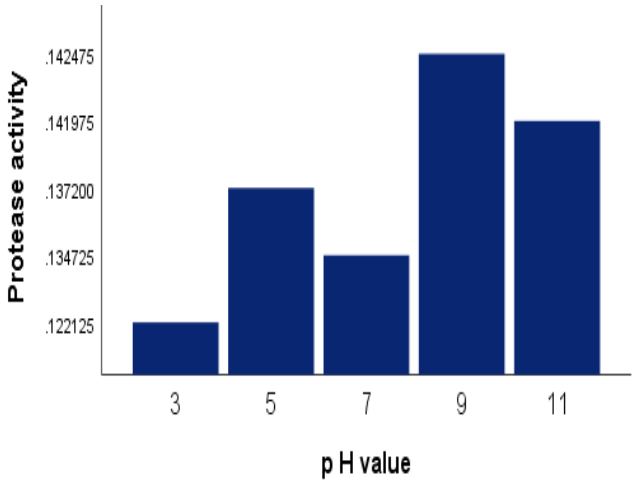

Figure 4b**:** Effect of pH on protease activity of produced by *Bacillus tropicus* PW10^{-2}A2

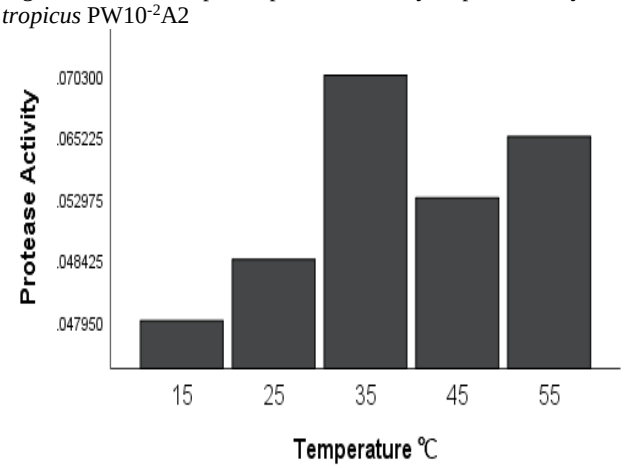

Figure 5a: Effect of temperature on protease activity of *Bacillus tropicus* PW10^{2}A2

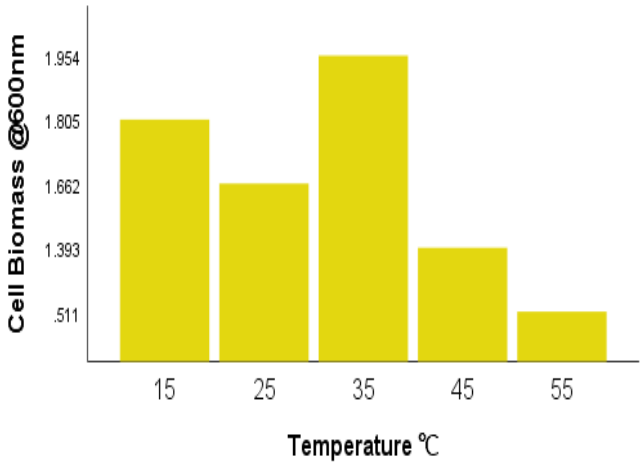

Figure 5b: Effect of temperature on growth of cell biomass by *Bacillus tropicus* PW10^{2}A2

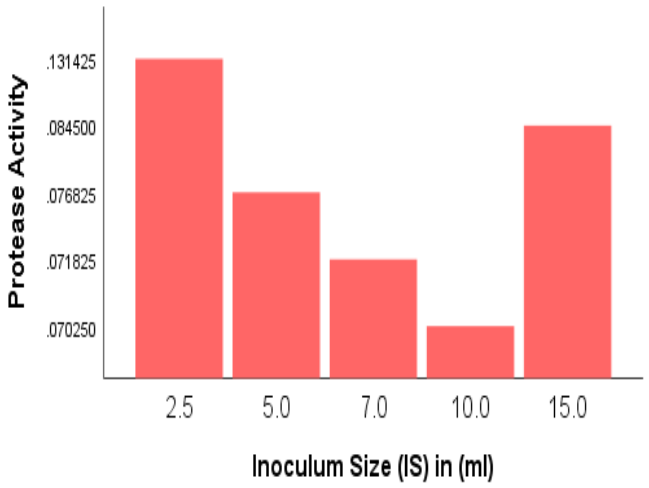

Figure 6a**:** Effect of inoculum size on protease activity by *Bacillus tropicus* PW10^{2}A2

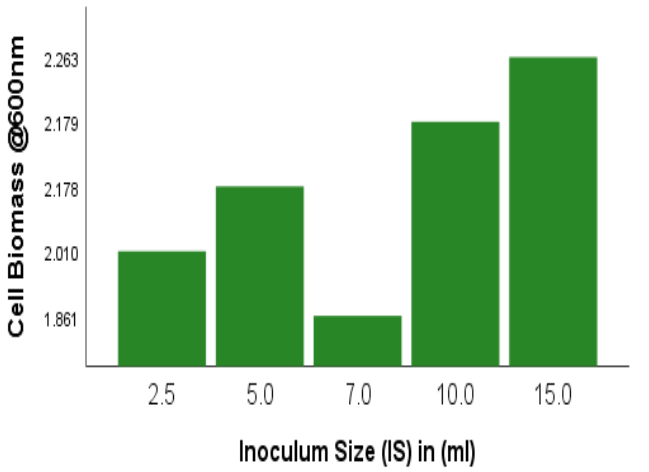

Figure 6b**:** Effect of inoculum size on growth of cell biomass by *Bacillus tropicus* PW10^{2}A2

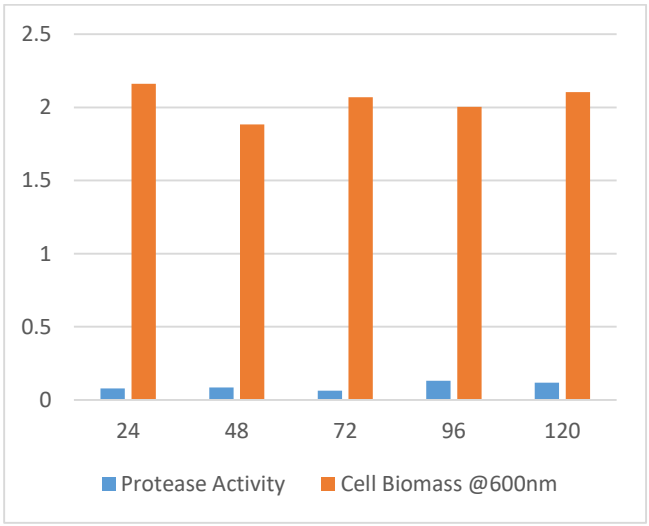

Figure 7: Effect of inoculum age on growth and production of alkaline protease by *Bacillus tropicus* PW10^{2}A2

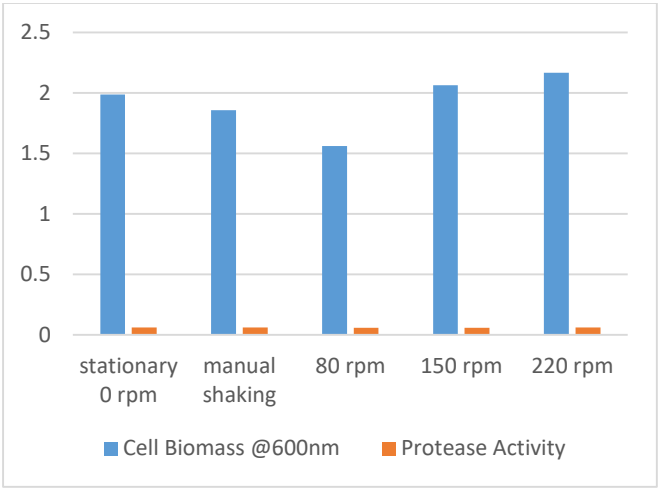

Figure 8**:** Effect of agitation speed on growth and production of alkaline protease by *Bacillus tropicus* PW10^{2}A2

4. Discussion

The successful isolation of protease-producing bacteria from poultry farm water highlights the ecological richness of such environments as reservoirs of industrially important microorganisms. The isolate PW10^{-2}A2 exhibited the highest proteolytic activity (13 mm ZOH), confirming its strong extracellular enzyme secretion capability (Table 1). Larger hydrolysis zones are directly correlated with higher protease production, consistent with previous findings (Sharma *et al*., 2019; Alisa *et al*., 2020; Anukam *et al*., 2020a; 2020b; Umeh *et al.*, 2020; 2021; Umennadi *et al*. 2026; Uba and Umennadi (2026).

Biochemical characterization confirmed the isolate as *Bacillus tropicus*, a species known for its metabolic versatility and enzyme-producing potential (Table 2). The organism's ability to hydrolyze multiple carbohydrates and proteins indicates adaptability to diverse substrates, which is advantageous for industrial fermentation processes as published by several authors (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).

Hence, the nutritional (carbon, nitrogen (inorganic and organic) sources) and physical factors (temperature, pH, agitation, inoculum size and inoculum age (days) were optimized to obtain high levels of protease by PW10^{-2} A2 in basal medium. From the present study, *Bacillus tropicus* Pw10^{-2}A2 that was isolated from poultry farm source proved its beneficial ability to produce alkaline protease in a very affordable optimized conditions for enzyme activity maintained at pH 9, incubation temperature of 35 ^{0}C, 220 rpm agitation speed, 96 h inoculum age, 2.5 mL inoculum size, rice bran as carbon source, beef extract or NH_4NO_3 as inorganic or organic nitrogen sources. Similar work was done by Prabhavathy *et al.* (2013) where they used very cheap and easily available sources of protein and carbon to optimize

conditions necessary for better production of metabolites (enzymes) by ubiquitous microorganisms, which is one of the significances of this present study.

In order to determine the optimum incubation time for maximum enzyme production, 100 mL of the growth medium was inoculated with 2.5 mL of 24-h-old $PW10^{-2}$ A2 culture. The inoculated flasks were treated for different time intervals at 35 °C and 150 rpm. Optimum enzyme production was obtained after 96 h of incubation (Figure 1). The observation made in this work agreed with the result of Muhammad *et. al.* (2008) that maximum enzyme production was observed after 96 h with alkalophilic *Bacillus* sp. isolated from natural habitats.

When glucose in basal medium (glucose 0.1g, peptone 1g, yeast extract 0.02g, $MgSO_4$ 0.01g, $CaCL_2$, K_2HPO_4 @ pH 9) was replaced by various carbon sources (such as cellulose, sucrose, maltose, starch, glycerol, cassava peels, yam peels, rice bran), the carbon source preference ranking of $PW10^{-2}A2$ enzyme production was determined as Rice bran was seen to be the best preferred carbon source with 0.1567 µmol/mL protease activity, 6.268 micromoles of tyrosine released; there is not much increase in enzyme production compared to basal (control) medium. On the other hand, maltose (0.1085475 µmol/mL, 4.341 micromicromoles of tyrosine released) caused reduced protease synthesis. Bacterial growth was not parallel with enzyme production (Figure 2). This supports previous studies highlighting agro-industrial wastes as cost-effective substrates for enzyme production (Razzaq *et al*., 2019).

Nitrogen sources also was seen to have great effect on enzyme production. As a result, effects of various organic and inorganic nitrogen sources on production of protease were investigated. Inorganic nitrogen sources ranged from NH_4 NO_3, KNO_3, $NaNO_3$ and organic nitrogen: casein, skimmed milk, beef extract, corn steep, cow dung, plantain peels. Results showed that the best organic nitrogen source for protease production by $PW10^{-2}A2$ was beef extract, and enzyme yield was 0.1671 µmol/mL compare to basal medium 0.1420175 µmol/mL. On the other hand, inorganic nitrogen source that did excellently was NH_4 NO_3 0.17435 µmol/mL indicating that inorganic nitrogen sources can enhance enzyme synthesis more effectively than some organic sources. This may be due to the rapid assimilation of inorganic nitrogen by microbial cells. Bacterial growth was not parallel with enzyme production **(**Figures 3a and 3b**).**

The effect of pH on protease production was investigated also, the *Bacillus tropicus* $PW10^{-2}A2$ isolate was investigated with various basal culture media with pH ranging from 3, 5, 7, 9 and 11. The highest levels of protease production was achieved in the culture grown at pH 9, thereby indicating the isolate can be classified as alkalophilic microorganism (Figures 4a and 4b). The optimal pH was 9.0. In the alkaline conditions (pH 10.0 – 12.0), the relative activity gradually decreased from 0.142475 to 0.141975 µmol/mL compared to the protease activity at pH 9.0. This finding was in accordance with the published works of Lich *et. al.* (2022) and Muhammed *et. al.* (2008) research outcome.

Temperature is one of the most critical parameters to be controlled in any bioprocess or enzyme production. This factor was investigated with temp. ranging from 55, 45, 35, 25 and 15 ^oC. The effect of temperature on alkaline protease production revealed that maximum yield by the *Bacillus tropicus* $PW10^2A2$ (0.0703 µmol/mL) was obtained at 35 °C (Figures 5a and 5b). As the temperature increased, a dramatic decrease in growth and enzyme activity was observed and agreed with the finding of Özdemir *et al.* (2020). The findings from this present work in Figures 5a and 5b also support the previous report of Muhammed *et. al.* (2008) that most alkaline *Bacillus* strains are mesophilic types with optimal temperature ranging from 30 to 37 ^0C.

This work also proved that the consumption of nutrients gradually depends on the bacterial population and the abundant yield in alkaline protease production isn't dependent on the largeness of the inoculum size. Hence, inoculum size is crucial in optimizing production and can result to nutritional stress affecting product formation (Lich *et. al.,* 2022). The maximal protease activity (the best) achieved at 0.131425 µmol/mL with inoculum size of 2.5 mL as seen in Figures 6a and 6b. The inoculum age is also of importance when using mesophilic organisms due to the relatively low growth rate. In this study, the maximum enzyme production was observed with 96 h culture (Figure 7). All these findings indicate that inoculum age and size (concentration) have great effect on cell growth and protease production, depending on the properties of the strain.

Agitation leads to better distribution of substrate, nutrients and oxygen in medium. The effect of different agitation speeds on the protease production and growth of *Bacillus tropicus* $PW10^{-2}A2$ strain was observed by incubating the fermentation medium at different rpm speed: 0 rpm, manual rpm (30 mins daily), 80 rpm, 150 rpm and 220 rpm (Figure 8). The optimum agitation rate for protease production observed from this study is 220 rpm (Figure 8). The smallest alkaline protease activity (0.0569 µmol/mL) and cell growth (biomass OD @ 600: 1.559) were observed at 80 rpm, suggesting the limitation of oxygenation as well as compromised homogenization of the medium factor; still, alkaline protease production and growth of *Bacillus tropicus* strain $PW10^{-2}A2$ increased as agitation speed increased. Based on the results, 220 rpm was determined to be the optimum agitation speed for maximum yield (0.0604975 µmol/mL). Previous publications reported that 180 and 220 rpm are the most suitable agitation speeds for enzyme production by most *Bacillus* strains (Nadeem and Qazi, 2010).

One-way ANOVA demonstrated that incubation time, carbon and nitrogen sources, pH, temperature, inoculum size, inoculum age, and agitation significantly influenced protease production ($p < 0.05$). Maximum enzyme yield was obtained under optimized conditions (96 h, pH 9, 35 °C, rice bran, NH_4NO_3, 2.5 mL inoculum, 220 rpm). Correlation analysis

indicated that enzyme production was not strictly growth-associated (r = 0.42).

5. Conclusion

This study successfully isolated and characterized a potent alkaline protease-producing bacterium identified as *Bacillus tropicus* PW10^{-2}A2 from poultry farm water. The isolate demonstrated high proteolytic activity and metabolic versatility. Optimization of fermentation parameters revealed that maximum enzyme production occurred at 96 h, pH 9, 35 °C, with rice bran and NH_4NO_3 as optimal carbon and nitrogen sources, respectively Notably, enzyme production did not directly correlate with biomass, indicating that protease synthesis is regulated independently of cell growth. Overall, the systematic optimization of fermentation parameters resulted in a substantial improvement in protease yield, demonstrating the feasibility of developing an efficient and cost-effective bioprocess for large-scale production.

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