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Optimization, Characterization, and Detergency Application of Alkaline Protease Produced by *Bacillus haynesii*-A6 Using Agrowastes

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Abstract

Most industrial processes rely heavily on chemical reactions, which can be dangerous to human health and produce toxic byproducts that are released into the environment. Hence, there is a need for a healthier, more eco-friendly option, which microbial enzymes offer. Bacterial isolation was performed using the pour-plate method, and the isolates were screened for proteolytic activity. Identification of the selected isolate was carried out by cultural, biochemical, and molecular techniques. Isolate A6 was identified to be a species of Bacillus. The 16S rRNA gene sequence showed it to be closely related to B. haynesii. The best parameters for protease synthesis included pH 8.0, 40 °C, and plantain and soybean bran as carbon and nitrogen sources, respectively. Total protein content ranged from 39.7 mg/mL in crude to 7.05 in dialysis, while specific enzyme activity increased from 3.48 to 6.29 U/mL, a fold increase from 1.00 to 1.79 as purification increased. Total enzyme activity decreased from 412 mg/mL in crude enzyme to 132.0 mg/mL in dialysis. A molecular mass of 36.0 kDa was determined for the partially purified enzyme. The purified enzyme was active at 40 °C, pH 8, with Mg^{2+} and Na^{+} as metal ions, and was stable in the presence of surfactants, EDTA, organic solvents, and oxidizing agents. Our report showed that B. haynesii-A6 can be used to produce alkaline protease from cheaper, readily available agro-wastes. The protease obtained in this study can be used in detergent formulations and other biotechnological applications.

Keywords: Agro-wastes, Alkaline Protease, Bacillus species, Detergent industry, Ion Exchange Chromatography

INTRODUCTION

Proteases are a category of enzymes that hydrolyze peptide linkages within proteins, and microorganisms primarily produce them. However, they are found in all living organisms. Proteases are grouped into four classes according to their active sites. However, López-Otín & Bond, (2008) classified proteases into six categories based on their mechanisms of action or catalysis. The groups are glutamic acid, aspartic acid, cysteine, serine, threonine, and metalloproteases. Other criteria used to classify proteases include optimal pH and temperature (Bizuye et al., 2014). Proteases are grouped according to the functions possessed, the location of the bond by peptide, as well as whether they are acid or alkaline (Razzaq et al., 2019). Despite their numerous applications, protease production costs remain high, driven by ever-increasing demand for industrial and biotechnological applications (Gimenes et al., 2021). The reduction in the cost of enzyme production using cellulosic agricultural wastes has been a welcome development. Proteases are naturally present in microorganisms, plants, and animals (Naveed et al., 2021). *Bacillus* species are well-documented to produce large quantities of proteases that are stable under extreme environmental conditions (Contesini et al., 2018). However, alkaline proteases of microbial origin, especially those obtained from *Bacillus*, are most attractive for industrial applications. They can be active under conditions of higher pH (Razzaq et al., 2019). They represent the most sought-after hydrolytic enzymes worldwide (Contesini et al., 2018). They are produced extracellularly and are among the most studied enzymes from microorganisms due to their wide applications (Razzaq et al., 2019; Ullah et al., 2022).

Microorganisms occupy a unique position in the ecosystem and are found virtually everywhere in the environment, such as water, soil, and air. But they are predominantly present in the soil. They are actively involved in life activities, such

as the decomposition and recycling of organic and inorganic matter in the environment (Ilesanmi et al., 2020). Microorganisms are known to be prolific producers of proteases and other hydrolytic enzymes. They do not require much space for optimal growth, and they are robust. They grow and produce their metabolites, including protease, within a short time. The stability and activity of the enzymes were observed under different extreme conditions, including pH, temperature, and inhibitors (Bekele, 2020).

Furthermore, chemicals of various types are used in most industries, which affect human health. Due to their toxicity effects on living organisms, they are regarded as environmentally unfriendly. The replacement of synthetic chemicals with natural products derived from microorganisms has been a central focus of many researchers today (Razzaq et al., 2019). Such replacement involves the use of proteases, which are used in the detergent, food, cosmetics, leather, and textile industries, as well as in the medical and pharmaceutical sectors, and in wastewater treatment (Majeed et al., 2024; Naveed et al., 2021; Razzaq et al., 2019).

Nigeria is an agrarian country with extensive planting activities, which subsequently generate agro-wastes, either from harvesting or processing farm produce. These agro-wastes are either burnt in the open or left to natural decay, which may degrade the environment and serve as breeding grounds for rodents and disease-causing parasites and vectors. Most of these agro-wastes are not properly managed or utilized for economic purposes (Oyegoke et al., 2023). Nigeria is one of the highest producers of Bambara nut and soybean in sub-Sahara Africa (Hillocks et al., 2012; Khojely et al., 2018). Similarly, Nigeria is a major producer of plantain and banana in West Africa, alongside countries such as Côte d'Ivoire and Ghana (Olumba & Onunka, 2020). But the agro-wastes generated by these plants are usually left to decompose in the environment. Hence, we decided to use some agro-wastes, such as plantain and banana peels, soybean bran, and Bambara nut bran, in this study for protease synthesis, which could lower production expenses.

Presently, we reported the isolation of a *B. haynesii*-A6 strain with remarkable alkaline protease activity, optimized conditions for protease production, and characterized the protease produced. The enzyme, when added to detergent, effectively removed blood stains from white cloth.

MATERIALS AND METHODS

2.1 Samples collection

Ten water samples were collected from the River Gongola within the Yola North Local Government Area of Adamawa State. (Latitude 9 2610° N and Longitude 12.4328° E). They were collected in sterile sample bottles, placed on ice, and transported to the Laboratory for study.

2.1.1 Isolation and screening for protease activity

One milliliter (1.0 mL) of each water sample was mixed with 9 mL of sterilized water. Hundred microliter (100 µL) of mixed samples from 10^{-3}, $10^{-4,}$ and 10^{-5} were inoculated on sterilized nutrient and starch agar and incubated at room temperature. Pure cultures of the isolate were placed on slants of nutrient agar and stored at 4 °C in a fridge until needed (Ilesanmi et al., 2020; Pant et al., 2015).

All isolated bacteria were evaluated for proteolytic activity by culturing each on skim milk agar, starch-casein agar, and compounded nutrient agar with gelatin. All the media used were sterilized in an autoclave at 121 °C for 15 min. The plates were incubated at 30 °C for 3 to 7 days, and the zones of hydrolysis around the growth were noted. The hydrolysis zone was measured with a metric ruler and recorded in millimeters (Pant et al., 2015).

2.2 Characteristics of protease-producing bacteria

The selected bacteria were identified by their cultural, biochemical, and molecular traits. Cultural expressions in the media were noted. Molecular identification was performed and briefly explained. DNA was extracted using the Norgen DNA extraction kit (Model 24700, Norgen, Canada) according to the manufacturer's instructions. The pure 16S rRNA gene was subjected to PCR for its amplification with forward primer-243 5'-GGATGAGCCCGCGGCCTA-3' and reverse primer A3 5'-CCAGCCCCACCTTCGAC-3'. The primers are universal. The PCR cycle involves one cycle of DNA helix separation at 95 ºC that lasts for 5 min; then cycles of 35 cycles that involve the separation of the DNA double helix structure for 30 sec at 95 ºC, recombination of the separated strands at 49 ºC for half a minute, and then for 1 min, 30 sec, and at 72 ºC, elongation takes place. The last cycle of lengthening lasted for 10 min at 72 ºC. The integrity of the pure DNA was visualized by gel electrophoresis using NEB's

FAST DNA Ladder. The amplified PCR product was sequenced using the method of Sanger & Coulson (1975). The sequencing was carried out at the Inqaba Laboratory in Pretoria, South Africa. Each sequence was trimmed and analyzed for homology using the BlastN search program.

2.2.1 Biochemical characteristics of the selected proteolytic isolates

The ability of the proteolytic isolates to ferment the following carbon sources: glucose, sucrose, fructose, galactose, maltose, lactose, mannitol, D-xylose, soluble starch, and meso-inositol was tested. Similarly, selected isolates were assessed for their ability to utilize casein, urea, starch, gelatin, and citrate, and for the production of oxidase and catalase, as well as for the release of hydrogen sulfide from TSIA agar. The methods used for the biochemical study were as described by Fawole & Oso (1988).

2.3 Effects of production factors on protease

2.3.1 Sources of carbon

The effect of different carbon sources, including maltose, sucrose, fructose, glucose, banana, and plantain peels, on protease synthesis by the selected bacterium was assessed by substituting each source in turn as a carbon and energy source. One milliliter of the inoculum was added to a 100 mL medium containing 1.0 % w/v carbon source. Incubation was done at 30 °C for 96 h. The protease activity, as well as its overall protein content in the supernatant free of cells, was measured using a spectrophotometer at 660 and 595 nm, respectively (Sharma et al., 2014).

2.3.2 Sources of nitrogen

The impacts of different nitrogen sources, such as yeast and malt extracts, beef extract, peptone, casein, soybean bran, Bambara nut bran, NH_4SO_4, and $NH_4H_2PO_4$, on protease synthesis by the selected isolate were assessed. Each nitrogen source was introduced singly into the production medium at 1% w/v. The result of the best carbon source was maintained. Enzyme activity and protein content were evaluated according to the procedure of Sharma et al. (2014).

2.3.3 pH

The carbon- and nitrogen-adjusted basal medium was calibrated to pH 5, 6, 7, 8, 9, 10, and 11 to assess the effects of different pH on protease synthesis and activity. The medium was inoculated with *Bacillus haynesii*-A6, followed by incubation for 96 h at 30 °C. The protease activity and protein content were evaluated using a spectrophotometer (Sharma et al., 2014).

2.3.4 Fermentation period

The effect of fermentation periods of 24, 48, 72, 96, and 120 h on protease activity produced by Bacillus haynesii-A6 was assessed after the medium was formulated with previously determined production parameters. Incubation took place at 30 °C until each experiment was terminated. The method described by Sharma et al. (2014) was used to determine how active the enzyme was as well as its protein content.

2.3.5 Effect of temperature

The best values obtained for the previously determined production parameters that included the sources of carbon and nitrogen, the pH, temperature, and fermentation period were maintained in the basal medium, and production temperatures were adjusted to 20 ºC, 30 ºC, 40 ºC, 50 ºC, and 60 ºC. The fermenting medium was incubated at different temperatures. Protease activity and protein content were done as earlier described by Sharma et al. (2014).

2.4 Production and extraction of protease

One milliliter of *Bacillus haynesii* A-6 was introduced into 100 mL of modified production broth that contained (w/v) powdered plantain peels-0.1, $CaCl_2$ -0.01, K_2HPO_4-0.05, $MgSO_4$-0.01, soybean bran-0.1, pH (7.4). Fermentation was carried out at 40 °C for three days.

2.4.1 Assaying for protease activity

Two milliliters of the fermented medium were centrifuged for 15 min at 4 °C and 8000 rpm. The supernatant was used to measure protease activity based on the description of Carrie Cupp-Enyard as reported by Sharma et al. (2014). Briefly, two (2) test tubes were labeled T for the test and B for the blank. Five milliliters (5mL) of casein solution (0.65%) dissolved in 50mM potassium phosphate buffer of pH 7.5 was added to the test tube labeled T. One milliliter of crude enzyme extract was added to the test tube labeled T. The enzyme was not added to the blank B-test tube. The test and blank test tubes were incubated at 40 °C for 30 minutes to allow an enzymatic reaction to occur. 5 mL of TCA (110 mM) was added to both test tubes to stop the enzymatic reaction. One milliliter (1 mL) of crude enzyme extract was added to the

B-test tubes to bring the total volume to 11.0 mL. The solution from both test tubes was filtered through filter paper, Whatman No. 1. Two milliliters each of the test and blank were transferred to two new test tubes, followed by the addition of 5 mL $NaCO_3$ (500 mM) and a two-fold diluted Folin-Ciocalteu phenol reagent. The mixture was kept in a dark room at room temperature for 30 min for the formation of the blue color. The amount of tyrosine released after the enzymatic reaction was determined at 660 nm against a blank using the tyrosine standard. A standard curve was prepared by measuring tyrosine concentrations ranging from 27.5 to 275 µM/mL. A unit of protease was defined as the amount of enzyme that liberates 1 µM tyrosine per 60 sec at 40 ºC and pH 7.5.

2.4.2 Determination of protein concentration

The amount of protein in the crude enzyme extract was measured by the Bradford method, using bovine serum albumin (BSA) as the standard. Coomassie Brilliant Blue G-250 (CBB) was prepared by dissolving CBB G-250 in 50 mL of 95% ethanol. 100 mL of phosphoric acid was added and made up to 200 mL. The CBB G-250 binds to protein, and the color changes from light green to blue. A spectrophotometer was used at 595 nm to measure absorbance (Bradford, 1976).

2.5 Partial purification of protease

Enzyme precipitation was carried out by slowly adding ammonium sulfate (75%) to the fermented broth, followed by filtration through Whatman No. 1 filter paper. The precipitated protein was recovered by centrifugation at 5000 rpm for 30 min at 4 ºC. The precipitate was then dissolved in 0.5 mM/L phosphate buffer, pH 7.4. Partial purification was carried out using a dialysis tube for 12 h in a phosphate buffer of pH 7.0. The supernatant was tested for protease activity and characterized (Sharma et al., 2014).

2.6 Partially purified protease characterization

2.6.1 Molecular weight determination of the protease using SDS-PAGE

To determine the molecular size of the partially purified protease, the procedure Laemmli, (1970) was adopted. The dialyzed protease was added to a phosphate buffer (0.5 mol/L) at pH 7.5. The supernatant was introduced to a column packed with Sephadex G-200. With the same buffer at 20 mL/h, Enzyme fractions were collected at 20 mL/h using the same buffer to elute the column. The fractions with high protease activity were pooled and used to determine molecular weights by SDS-PAGE. The separating gel was loaded with the active fraction in 1.2 % (w/v) and 1:2V stacking. The gel was stained with Coomassie blue, making the bands visible. The molecular band size was estimated by comparing the band position with that of the standard markers.

2.6.1 Activity and stability of protease at different pH

The influence of pH on the partially purified protease was assessed at varying pH values of 5, 6, 7,8, 9, and 10 at 40 °C for 10 min using different buffer systems: 0.1M sodium phosphate buffer (6.0-7.0), tris-HCl buffer (pH 8.0), and glycine -NaOH buffer (pH 9.0 –10.0). Casein 1% (w/v) was used as the substrate. The pH stability was assessed by pre-incubating the purified enzyme, without substrate, at the pH values indicated. Residual proteolysis was assessed as described by Sharma et al. (2014).

2.6.2 Influence of temperature on protease activity and stability

The protease activity and its stability at 20 °C, 30 °C, 40 °C, 50 °C, and 60 °C, at a constant pH of 8.0, were determined. The thermal stability of the partially purified protease was tested at each temperature for 5 h. The activity of the protease was assessed by using the description of Sharma et al. (2014).

2.6.3 Influence of metal ions on protease activity

The partially purified protease was assessed for its activity in the presence of different ionic concentrations of 5 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, $Cu^{2+,}$ and Na^{+}. The enzyme at pH 8 was incubated at 40 °C for 20 min. Casein was added as substrate. The procedure of Sharma et al. (2014) was employed to determine the residual activity of the enzyme.

2.6.4 Effect of inhibitors on enzyme activity

Impacts of ethylenediaminetetraacetic acid (EDTA) and phenyl sulfonyl fluoride (PMSF) as

inhibitors were assessed on the activity of the partially purified protease. The protease was incubated along with the inhibitors (5.0 mM) and a buffer (phosphate) at pH 7.5 at 40 °C for half an hour. The control was also treated like the test, but with no inhibitor. The residual

activity of the protease was assessed using the method of Sharma & De (2011).

2.6.5 Influence of surfactants, oxidizing agents, and organic solvents on enzyme activity

The effects of surfactants (Tween-20, 80, and Triton X-100), sodium hypochlorite, and solvents (acetone, ethanol, and 1% methanol) on enzyme activity and stability were evaluated by incubating the enzyme for 60 min at 40 °C and pH 7.4 (Vijayaraghavan et al., 2012).

2.7 Application of the partially purified protease (De-staining)

The de-staining potential of the partially purified protease was assessed by immersing pieces of white cloth in blood for 15 minutes. It was then allowed to dry. Each of the blood-stained cloth was placed in a conical flask. The treatments were: flask 1 contained the blood-stained cloth, 100 milliliters of distilled water, and 1 milliliter of detergent solution (7 mg/mL); flask 2 contained 100 milliliters of distilled water, 4 milliliters of the enzyme, and the blood-stained cloth. However, flask 3 contained the blood-stained cloth, the partially purified enzyme, and the detergent prepared in 100 mL of distilled water, while the last flask (4) contained the blood-stained cloth in 100 mL of distilled water. All the treatments were incubated at 40 °C for 15 min. This was followed by rinsing with distilled water and drying. The blood-stained clothes were examined for bloodstains (Adinarayana et al., 2003).

2.8 Data analysis

Data were collected in triplicate and reported as the mean with standard deviation (Mean ± SD). The results were presumed to be significantly different at P values ≤ 0.05

RESULTS

3.1 Isolation and determination of protease activity

Twenty-six bacteria were isolated. Eight of the bacterial isolates exhibited remarkable proteolytic activity, ranging from 27±0.2 to 69±0.3 mm on starch casein agar. However, further screening showed that isolate A6 had the highest proteolytic activity against three proteins, including casein (46±0.23 mm), gelatin (34±0.15 mm), and skim milk (56±0.63 mm).

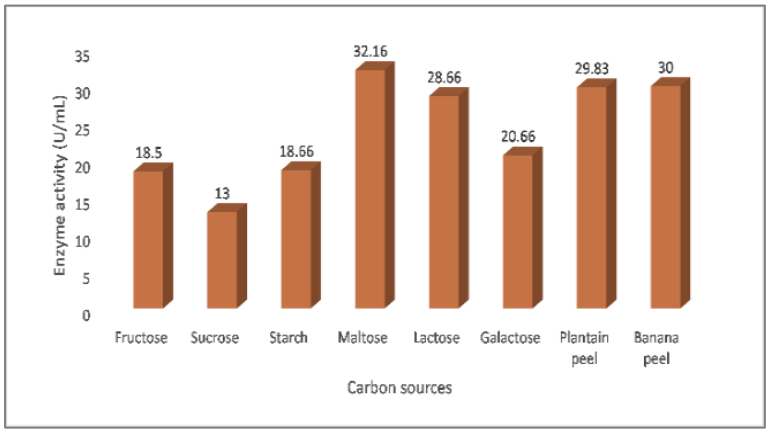

Figure 1a: Effects of Carbon sources on protease production by *B. haynesii*-A6

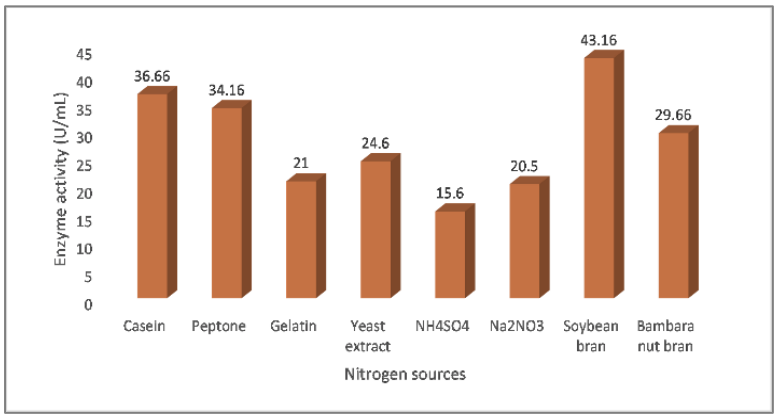

Figure 1b: Effects of Nitrogen sources on protease production by *B. haynesii*-A6

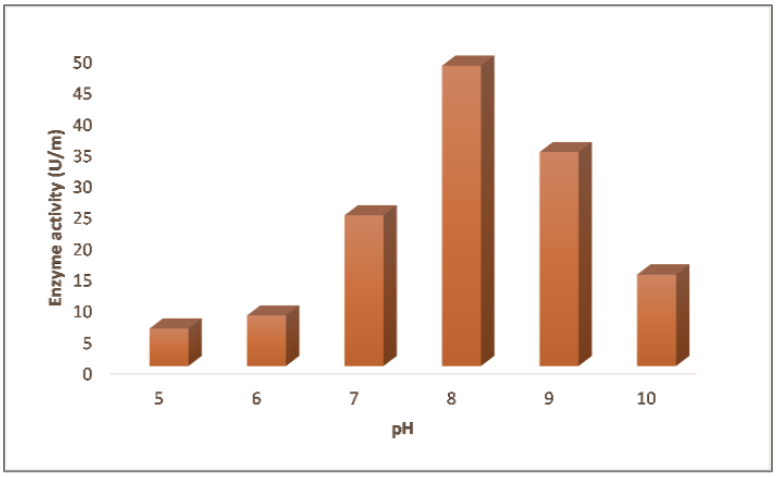

Figure 1c: Effects of pH on protease production by *B. haynesii*-A6

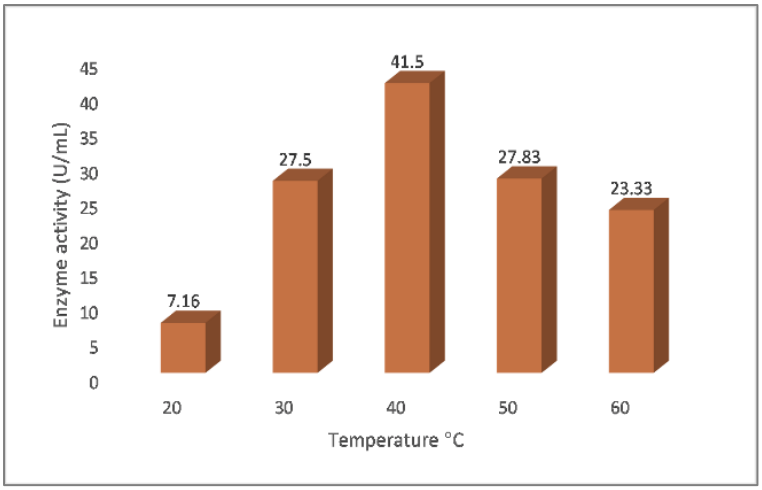

Figure 1d: Effects of Temperature on protease production by *B. haynesii*-A6

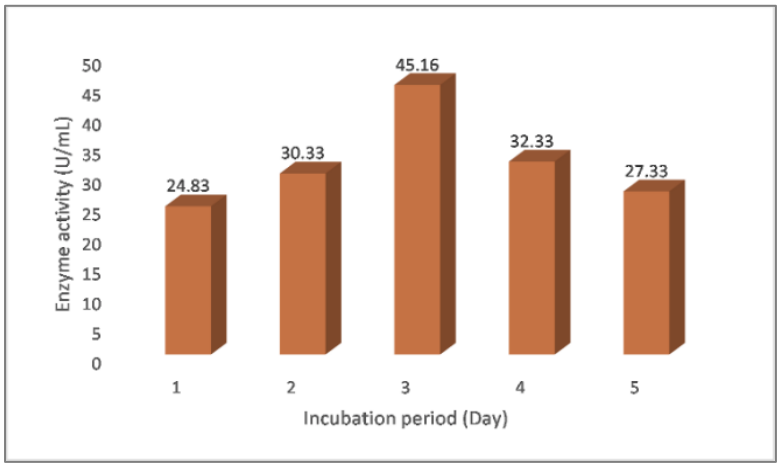

Figure 1e: Effects of Incubation time on protease production by *B. haynesii*-A6

3.2 Identification of selected bacterial isolate

3.2.1 Cultural, biochemical, and molecular identification

Isolate A6 had a smooth, shiny surface, with aerial and reverse side colors mostly between ivory/fussy white on nutrient and starch casein agars. The selected isolate utilized various sugars, including glucose, maltose, fructose, lactose, and galactose, as carbon and energy sources. However, it was unable to utilize sugars such as sucrose, mannitol, citrate, and D-xylose. Similarly, isolate A6 hydrolyzed gelatin and produced urease and catalase, but indole and oxidase activities were not detected.

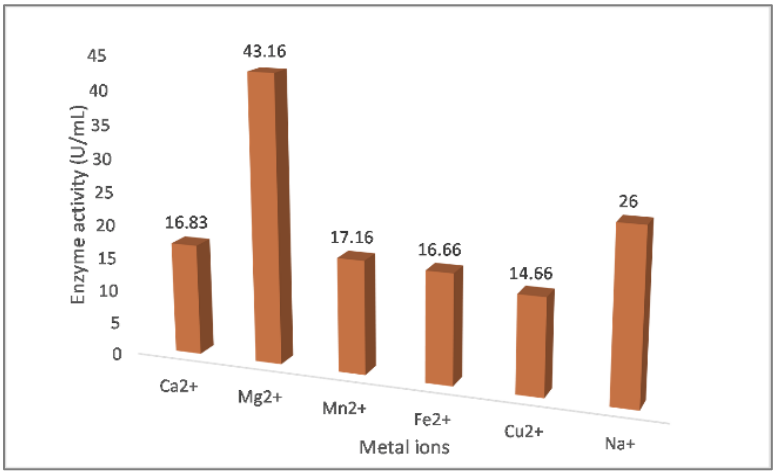

Figure 1f: Effects of Metal ion on protease production by *B. haynesii*-A6

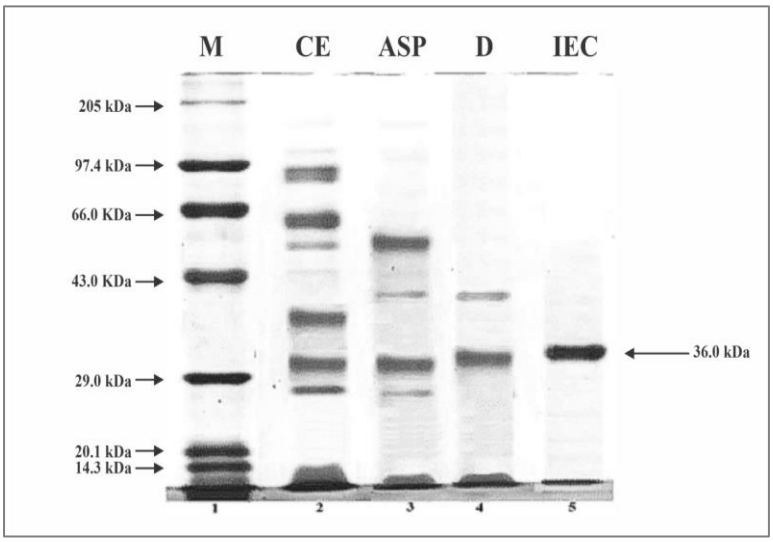

Figure 2 SDS-PAGE of protease produced by *B. haynesii*-A6

Keys: M= Marker, CE= Crude enzyme, ASP= Ammonium sulphate precipitate, D= Dialysis and IEC = Ion exchange chromatography

Table 1: Protease purification

Steps	Protein content (mg/mL	Enzyme activity (U/mL)	Total enzyme activity (mg/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Fold	% yield
Crude	39.46	37.50	412.50	118.38	3.48	1.00	100.00
(NH4)2SO4	19.03	24.00	264.00	57.09	4.62	1.33	64.00
Dialysis	11.50	18.50	203.50	34.50	5.89	1.69	49.3
IEC	7.05	12.00	132.00	21.15	6.24	1.79	52.00

key:IEC = Ion exchange chromatography

3.2.2 Molecular identification of isolate A6

The gene 16S rRNA was used in this study to further identify the selected isolate A6. The obtained sequence was subjected to the BLAST algorithm at the National Center for Biotechnology Information, NCBI. Isolate A6 was observed to belong to the genus *Bacillus* with a 97 % similarity index. Hence, the name *Bacillus haynesii*-A6. The 16S rRNA gene band was approximately 1.4 kb.

3.3 Effects of fermentation factors on protease production and activity

The influence of fermentation conditions, such as nitrogen and carbon sources, temperature, pH, and fermentation time, on the synthesis and

activity of the protease is presented in Figures 1a-1f.

3.3.1 Carbon sources

Carbon sources are known to provide the energy and carbon atoms required for the structural development of the fermenting isolate. Maltose, lactose, plantain, and banana peels gave considerable proteolysis. The least protease activity was recorded with sucrose. Using plant biomass, such as plantain and banana peels, also showed significant activity (Figure 1a).

3.3.2 Nitrogen sources

The highest enzyme activity of 43.16±0.3 U/mL was observed with soybean bran, followed by casein at 36.66±0.2 U/mL, while the least proteolysis was recorded with ammonium sulfate at 15.6±0.3 U/mL. The two plant biomasses tested in this study, soybean bran and Bambara nut bran, yielded good enzyme activity (Figure 1b).

3.3.3 pH

The influence of pH on protease synthesis and activity was strongest at pH 8, suggesting the likelihood of producing an alkaline protease. The enzyme activity reached its peak at pH 8.0 with a value of 48.16 U/mL and gradually decreased towards both extremes. The lowest protease production and activity were 6.10 U/mL at pH 5.0 (Figure 1c).

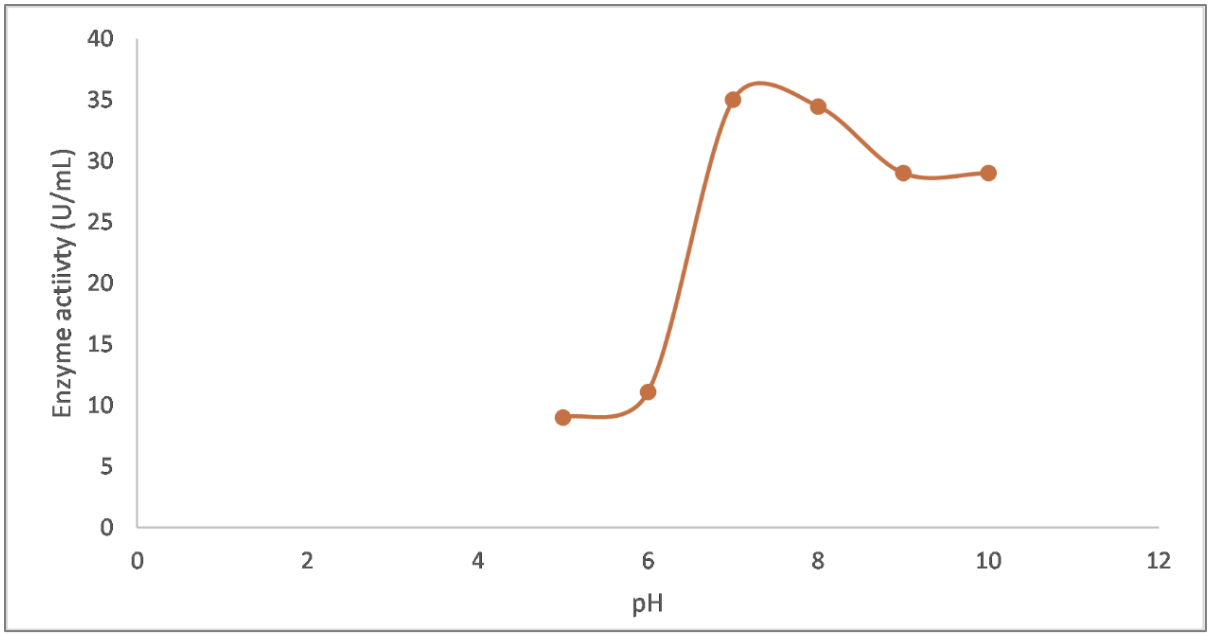

Figure 3a Influence of pH on protease activity

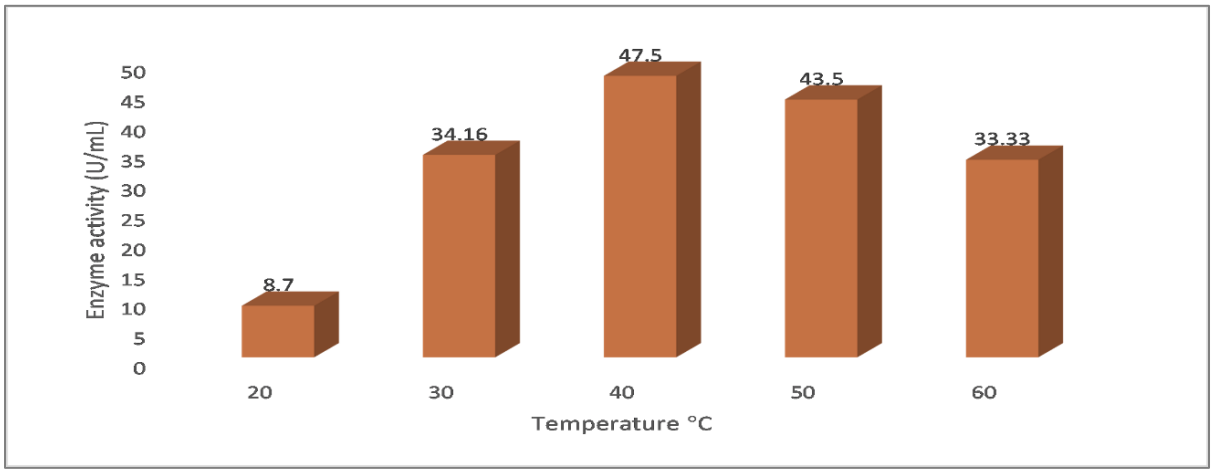

Figure 3b Impact of temperature on the activity of protease activity

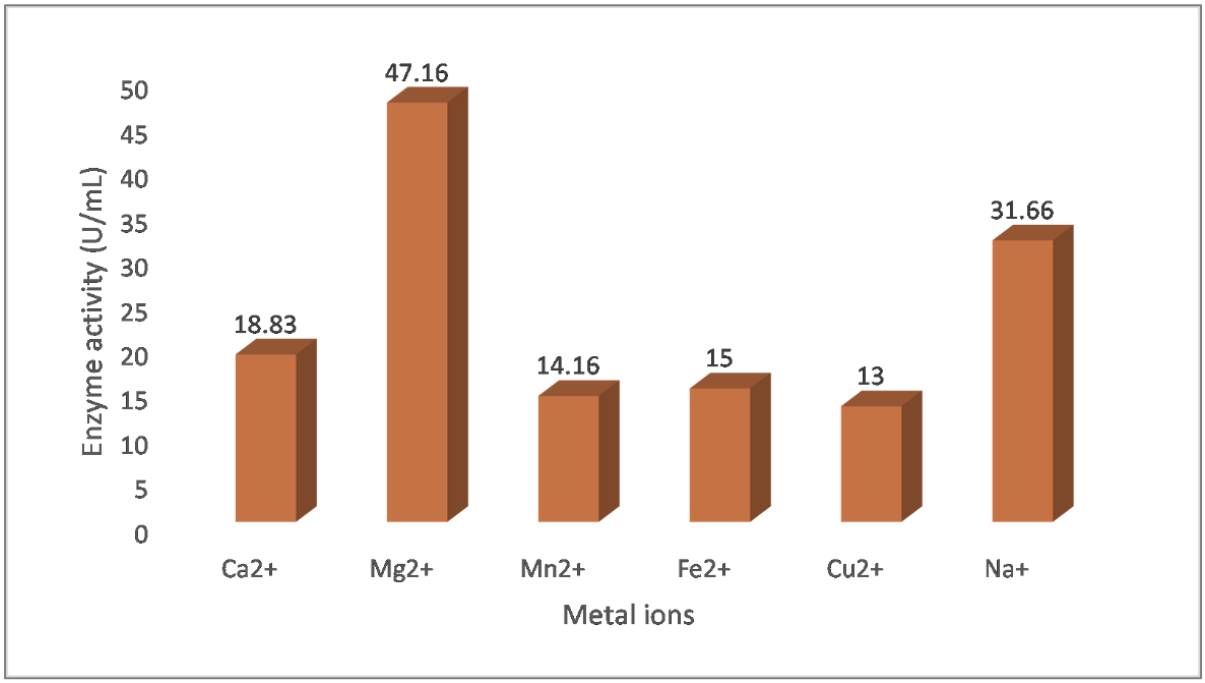

Figure 3c Influence of metal ions on protease activity

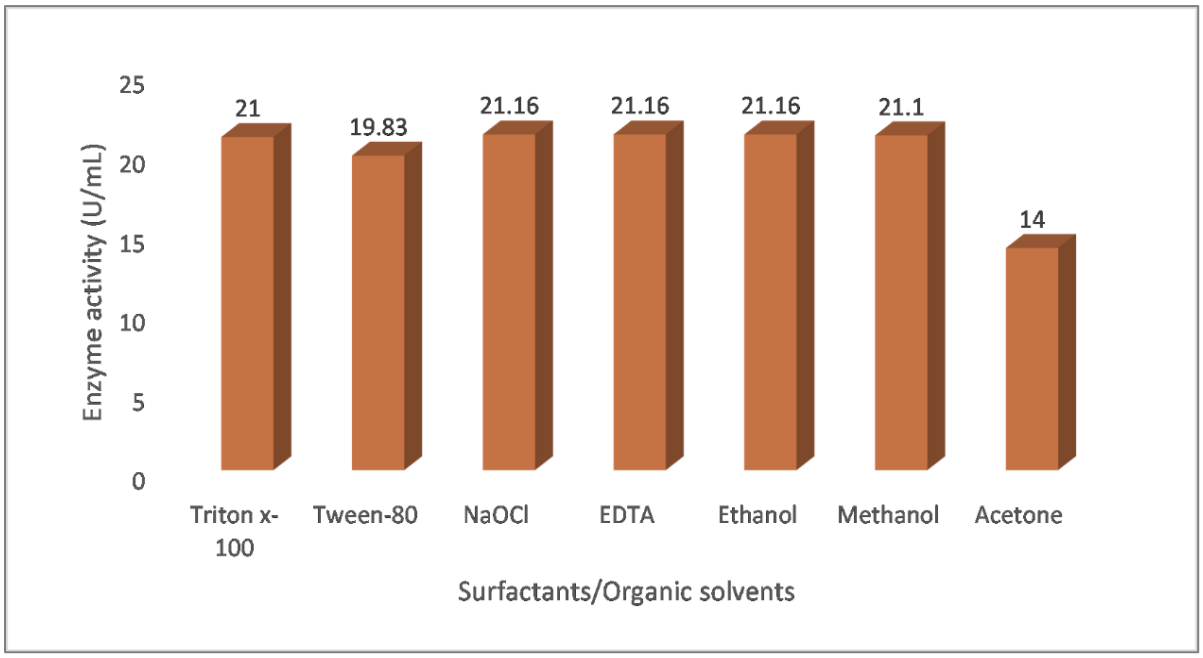

Figure 3d: Effect of surfactants, EDTA, and organic solvents on protease activity

3.3.4 Temperature

Protease was synthesized over a temperature range of 20 °C to 60 °C. However, the proteolysis peak occurred at 40 °C. Temperatures below and above 40 °C showed a remarkable decrease in enzyme activity (Figure 1d).

3.3.5 Effects of the incubation period

Incubation periods significantly affected the synthesis and activity of the protease produced by *B. haynesii*-A6. The highest enzyme activity, 45.16±0.3 U/mL, was observed on the 3rd day of fermentation. A significant decrease in enzyme activity was recorded before and after the 3rd day of fermentation (Figure 1e).

3.3.6 Effect of metal ions

The influence of different metal ions, such as calcium, magnesium, manganese, iron, copper, and sodium, on production and enzyme activity was examined. The highest proteolysis was observed in magnesium (43.16±0.3 U/mL),

followed by sodium (26.00±0.2 U/mL). The least proteolytic activity, 14.66±0.2, was observed when production was done with the addition of copper ions (Figure 1f).

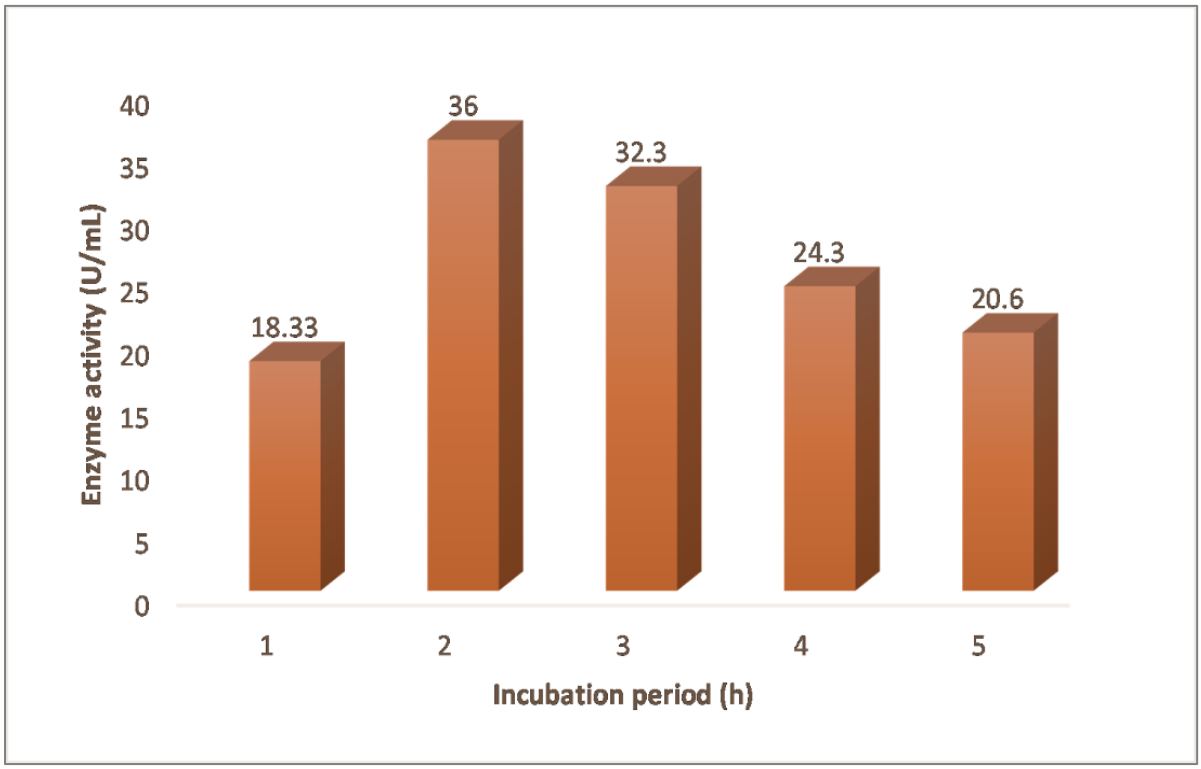

Figure 3e: Influence of incubation period on protease activity

1 2 3

Figure 4: De-staining activity of partially purified protease.

3.4 Partial purification

Fermented broth of *B. haynesii*-A6 was centrifuged, treated with ammonium sulfate, and partially purified by dialysis. The protein content was 39.46 mg/mL in the crude enzyme, while the lowest was observed in the Sephadex G-200-purified enzyme. Similarly, enzyme activity was at its peak in the crude enzyme (37.50 U/mL) and at its lowest in the Sephadex G200-purified enzyme (12.00 U/mL), with a 52.00% yield. There was a decrease in the enzyme's percentage yield. However, the specific activity and fold increased from 3.48 to 6.24 U/mg and from 1.00 to 1.79, respectively, along the course of treatment (Table 1).

3.5 Characterization of partial-purified protease

3.5.1 Molecular size determination

Both crude and partially purified protease preparations at different steps showed several bands corresponding to the standard markers. The crude protease showed six bands, which were reduced to four upon ammonium sulfate treatment. Further purification by dialysis reduced the number of proteins present, as shown in the Sephadex G200 (ion-exchange chromatography, IEC), with a single distinct band corresponding to 36.0 kDa (Figure 2).

3.6 Effect of pH, temperature, metal ions, inhibitors, and surfactants on protease activity

The effects of pH on partially purified protease showed optimal activity of 35.00 U/mL between pH 7 and 8, while the lowest enzyme activity was 9.00 U/mL at pH 5. The protease produced by *B. haynesii*-A6 appeared to be alkaline (Figure 3a).

The temperature had a significant influence on protease activity, which peaked at 40 °C (47.50 U/mL), while 43.50 U/mL was recorded at 50 °C (Figure 3b). A low temperature, such as 20 °C, did not yield good proteolytic activity, with a value of 8.70 U/mL.

Metal ions were observed to enhance proteolytic activity, with the highest (47.16±0.2 U/mL) activity observed with magnesium (Mg^{2+}). This was followed by sodium ion (Na^{+}) with 31.66±0.3 U/mL. Copper ions did not show a significant improvement in proteolytic activity (Figure 3c).

Proteolysis was not significantly improved when surfactants such as Tween-80 and Triton X-100, and oxidizing agents such as sodium hypochlorite, were added to the enzyme. Similar low enzyme activity was noted with EDTA and with organic solvents used. The proteolytic activity ranged between 14.00 and 21.16 U/mL, as presented in Figure 3d.

The partially purified enzyme retained its activity at 40 °C for 5 h, reaching a peak at the second hour; thereafter, activity decreased significantly (Figure 3e).

3.7 De-staining property of the partially purified protease

Treatment C showed the best de-staining activity, followed by Treatment B due to the synergistic effect of the detergent and the enzyme. Both treatments A and B partially removed the blood stain, whereas the blood stain in treatment D remained unremoved. Water could not remove blood stains from the white cloth (Figure 4).

DISCUSSION

Among enzymes with hydrolytic properties, proteases occupy a unique niche due to their commercial usefulness and the important roles they play in the physiology of living things. They are ubiquitous. Proteases are produced by microorganisms, mostly fungi and bacteria (Adetunji et al., 2023). The species of *Bacillus* have been extensively used in the production of alkaline proteolytic enzymes due to their rapid growth, extracellular secretion of enzymes, and ability to use cheap raw materials for enzyme synthesis (Adetunji et al., 2023; Ramalingam et al., 2022; Saggu & Mishra, 2017). However, the cost of raw materials for production and the continuous demand for proteolytic enzymes in the agricultural, pharmaceutical, medical, chemical, and textile, and environmental sectors of growing economies have placed greater pressure on researchers to screen for microorganisms capable of producing proteases using cheaper, readily available raw materials.

In this study, twenty-six bacterial isolates were evaluated for protease synthesis and activity. However, only eight exhibited proteolysis. Among the eight bacterial isolates, only isolate A6 hydrolyzed gelatin, casein, and skimmed milk. Cultural and biochemical characteristics showed that isolate A6 belongs to the genus *Bacillus*. An additional study of the 16S rRNA gene confirmed that isolate A6 belongs to the genus *Bacillus*. Hence, isolate A6 was tagged *Bacillus haynesii*-A6. Previous reports by Adetunji et al. (2023), Ramalingam et al. (2022), and Abdullah et al. (2022) showed that many species of *Bacillus* are versatile in the production of proteolytic enzymes. Most environmentally derived microorganisms harbor hydrolytic enzymes essential for their biochemical and physiological activities. These enzymes are used in the breakdown of organic materials in their vicinity (Adetunji et al., 2023).

In this study, *B. haynesii*-A6 utilized glucose, maltose, lactose, galactose, and citrate, but was unable to metabolize sucrose, mannitol, starch, citrate, and D-xylose. However, *B. haynesii*-A6 was able to hydrolyze gelatin, as well as production of urease and catalase, while it was negative for indole and oxidase. Our observations in the biochemical and sugar fermentation are similar to a previous report by Saggu & Mishra (2017) with slight differences, which could be attributed to the species of *Bacillus* used for the study, and the fermentation conditions. Besides these, *B. haynesii*-A6 was able to use agro-wastes such as plantain and banana peels, as well as soybean and Bambara nut brans. The use of these plant biomasses might considerably lower the production expenses of protease for industrial use. A report by Su et al. (2018) and Elumalai et al. (2020) showed that agro-wastes enhance protease production and could mitigate high production costs and help in the conversion of the agro-wastes to useful raw materials.

Carbon and energy sources like maltose, lactose, and plantain, along with banana peels, supported the growth as well as the production of protease by *B. haynesii*-A6. Similarly, casein, peptone, Bambara nut bran, and soybean bran. The potential of using agro wastes in this study produced cheaper alternatives for protease production by *B. haynesii*-A6. The versatility of *Bacillus* species in the breakdown of organic matter in their environment is controlled by the enzymes available to support their metabolic activities (Adetunji et al., 2023). Su et al. (2018) also reported the use of soybean meal for the production of protease. Soybean is known to be rich in proteins, carbohydrates, and minerals, and it enhances protease yield. Agro-wastes are rich in nutrients that might enhance the growth of microorganisms and the synthesis of their metabolites at lower cost (Elumalai et al., 2020).

The best temperature for protease production obtained in this study was 40 °C, and it is in line with previous reports by Ramalingam et al. (2022) and Saggu & Mishra (2017). However, others have reported varying temperatures for production that gave the highest enzyme activity such as 30 °C by Hakim et al., (2018), 32 °C Li & Fan, (2022), 37 °C by Hashmi et., (2022), 35 °C by Asha & Palaniswamy, (2018), 40 °C by Ramalingam et al., (2022), and 50 °C by Karray et., (2021). However, a lower temperature of 20 °C was reported by Mushtaq et al. (2023). The differences observed could be attributed to the fact that there are species of *Bacillus* that are thermo-tolerant and thermophilic; besides these, the environment where the bacterial isolate was obtained could contribute to the temperature growth requirement as well as the activity of the enzyme, as observed in Mushtaq et al. (2023) who obtained a protease that was active at low temperature of 20 °C. The *Bacillus* species used was isolated from a cold environment.

Similarly, pH 8.0 supported protease production by B. haynesii-A6, whereas most authors reported pH 8, 9, and 10 (Asha & Palaniswamy, 2018; Hakim et al., 2018; Hashmi et al., 2022; Ramalingam et al., 2022). Alkaline and neutral proteases are common in nature, as reported by several authors, and are most sought after for their catalytic properties and stability. The growth medium pH is a key component in determining the metabolism of the producing strain. pH influences the movement of materials in and out of the cells.

This study revealed that only magnesium (Mg^{2+}) significantly enhanced the growth of *B. haynesii*-A6 and proteolytic activity, with a value of 47.16±0.2 U/mL. Different metal ions have been reported to enhance the metabolic activities of microorganisms. Some act as cofactors by activating metabolic processes during redox reactions, donating or attracting electrons, positioning the substrate for the reaction, and stabilizing negative charges (Prejanò et al., 2020). In this study, magnesium ions (Mg^{2+}) improved protease production and activity, which is in agreement with the earlier report of Tennalli et al. (2022), Ramalingam et al. (2022), Saggu & Mishra (2017). Furthermore, different metal ions such as Ca^{2+} and Mn^{2+} (Asha & Palaniswamy, 2018; Ramalingam et al., 2022), and other metal ions like Zn^{2+} by Ramkumar et al., (2018), and Cu^{2+} by Tennalli et al. (2022) have been reported to enhance the development and synthesise of protease by different species of *Bacillus*.

A gradual increase in enzyme output and activity reached its peak (45.16±0.3) at 72 h, and thereafter reduction in enzyme activity. A similar incubation period of 72 h was reported by Hashmi et al. (2022) and Mushtaq et al. (2023), while some earlier publications showed that optimal production and activity of the protease produced was achieved by 24 h (Hakim et al., 2018) and 48 h, as reported by Asha & Palaniswamy (2018) and Hashmi et al. (2022). The rate at which the producing isolates multiply, along with the availability of carbon, nitrogen, or other limiting factors, could be responsible for the different periods of enzyme activity.

The partially purified protease produced by *B. haynesii*-A6 showed increased stability and activity from neutral pH 7 to alkaline pH 10, with the best proteolysis around pH 7.0 and 8.0. The report of Li & Fan (2022) showed that a pH of 7.0 was optimal for the stability of partially purified protease produced by *Staphylococcus simulans* QB7. Partially purified protease has been mentioned to be stable in activity within different pH values that ranged between pH 4 to pH 8 (Li & Fan, 2022), pH 5 to 12 (Ramalingam et al., 2022), pH 6 to 12 (Mushtaq et al., 2023), and 6 to 11 (Salim et al., 2023). Alkaline proteases appear to be better adapted to the metabolic and physiological conditions of the genus *Bacillus*.

Furthermore, the partially purified protease was observed to be steady between 30 °C and 60 °C, with the optimal proteolysis recorded at 40 °C, which was similar to the report of Hashmi et al. (2022) who worked with *Bacillus subtilis*. The highest protease activity has been reported

previously at 20 °C by Mushtaq et al. (2023), 50 °C by Li & Fan (2022), and 60 °C by Hashmi et al. (2022), who worked with *B. amyloliquefacien* while temperature ranges of 5 °C to 40 °C by Mushtaq et al. (2023), 30 °C to 60 °C by Hemsinli & Gurkok (2024), and 10 °C to 70 °C by Ramalingam et al. (2022) have been mentioned at which protease was found to be stable and active. This stability could be attributed to the structure of the enzyme, which is known to be enhanced by metal ions and surfactants.

Among the six metal ions used in this work, the highest proteolytic stability and activity were achieved with magnesium ion (47.16±0.2 U/mL), followed by sodium ion (31.66±0.3 U/mL). Improved stability and activity of partially purified protease by Na^+, Mg^{2+}, Ca^{2+}, and Mn^{2+} have been reported by Li & Fan (2022), Ramalingam et al. (2022), and Asha & Palaniswamy (2018), as well as Zn^{2+} Ramkumar et al. (2018) and Li & Fan (2022). Metal ions have been mentioned earlier as involved in the structural stability of the enzyme and in redox processes, where they function as electron donors (Prejanò et al., 2020).

Additionally, proteolysis was reduced from the highest (47.50±0.2) obtained without surfactants, EDTA, organic solvents, or an oxidizing agent to an average of 19.94±0.3 U/mL with Triton X-100, Tween-80, and organic solvents used. Similar reductions in enzyme activity were observed with EDTA and sodium hypochlorite. This clearly showed that these compounds did not improve proteolysis. However, the protease was stable and compatible with detergent, effectively removing blood stains. This is contrary to the reports of Karray et al. (2021) and Asha & Palaniswamy (2018), who mentioned stability and enhanced activity of partially purified protease in the presence of surfactants. Ethylene diamine tetraacetic acid disodium (EDTA) was mentioned to aid proteolysis of partially purified protease obtained from *simulans* QB7, a species of *Staphylococcus* (Li & Fan, 2022).

The partially purified protease studied retained the highest stability and activity for 2 h at 40 degrees Celsius, with a noticeable reduction in activity by the 4th h mark. Several authors have reported alkaline protease stability at different temperatures, over extended periods, and across diverse pH values. For instance, Alonazi (2024) mentioned the stability of the enzyme produced at 70 °C for 2h. Sodagar et al. (2024) recorded maximum stability and proteolysis at 50 °C along with pH 9.0. Furthermore, Hemsinli & Gurkok (2024) reported the optimum stability and activity of their protease at 40 °C and pH 9.0. This is similar to our observation in this study. The metal ions and the bonds between the structures of different proteases could be responsible for the differences in activity, either for a long period or at high temperatures.

The molecular sizes of the proteases produced by *B. haynesii*-A6 revealed that various sizes of proteases were simultaneously produced. The band sizes ranged from 28 kDa to 97.4 kDa. The purest protease obtained in this study showed a distinct band on ion-exchange chromatography and had a size of 36 kDa. Different molecular sizes in kDa have been mentioned by several researchers, such as 23.6 by Alonazi (2024), 35 by Ullah et al. (2022), 36 by Majeed et al. (2024), 38 by Farooq et al. (2021), and 47 by Li & Fan (2022). The likely reasons for the different band sizes reported could be the level of purification and the type of protease. In our study, the number of bands decreased as treatments progressed from the crude enzyme to the ammonium sulfate precipitated enzyme and the dialyzed enzyme, as shown in the ion-exchange chromatography.

The partially purified protease produced by *B. haynesii*-A6 was compatible with commercially available detergents. It completely removed blood from the the blood-stained cloth. This property has been mentioned earlier by Farooq et al. (2021) at twenty degrees Celsius, and other researchers such as Hemsinli & Gurkok (2024), Alonazi (2024), and Majeed et al. (2024) reported similar activity that protease effectively removed blood and chocolate stains. The removal of blood stains from clothes could be attributed to the stability of the partially purified to different surfactants, oxidizing agents, and solvents (Alonazi, 2024; Farooq et al., 2021; Majeed et al., 2024). The addition of protease to detergent formulations enhanced their cleaning efficiency, as reported, corroborating our findings in this study.

CONCLUSION

This study revealed the isolation and identification of *B. haynesii*-A6, its use in the production and characterization of the protease, and its detergency properties. The protease was produced from cheap, locally available agro-wastes such as plantain and banana peels, soybean and Bambara nut brans, thereby reducing the cost of protease production. The protease synthesized in this study was stable at pH 8 and a temperature of 40 °C. Its stability makes it attractive for

industrial, medical, and environmental use. When combined with detergent, it effectively removes blood stains from cloth, making it a promising additive material in detergent formulations and production

RECOMMENDATION

It is recommended that we explore the unexplored environments around us to identify microbial communities that could be beneficial to humans. Similarly, much crop production takes place in the Northern part of Nigeria, and the plant biomass produced is usually burnt, causing environmental pollution. However, these agro-biomass can be used as cheaper, readily available raw materials for the production of proteases needed for detergent and other biotechnological applications.

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CONFLICT OF INTEREST

None

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