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Investigating the Bioactivity of Extracellular Protease Produced by Bacterial Strains Collected from Different Soil in Egypt

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Abstract

Proteases are enzymes of considerable industrial importance, owing to their broad catalytic versatility across a wide range of applications. In this study, twenty-three bacterial isolates were obtained from cultivated soil samples. Among these, six isolates -designated B1, B7, D1, F2, G and H- exhibited notable proteolytic activity, as evidenced by clear zones of casein hydrolysis on skimmed milk agar and enhanced growth extending beyond the cultured disc boundary. Of these, isolate D1 demonstrated the most pronounced proteolytic activity, characterized by extensive degradation zones and vigorous peripheral growth. This isolate was taxonomically identified as *Alcaligenes faecalis* and subsequently deposited in the GenBank database under accession number PQ357195 (EG1-AMY).

Protease production was optimized by evaluating various submerged fermentation conditions. Following characterization of enzyme's activity profile, the protease was classified as an alkaline protease based on its optimal activity in alkaline pH. The highest enzymatic yield was achieved when all optimized parameters were combined in a single test, resulting in an approximately fourfold increase in protease activity compared to the original condition.

Partial purification of the enzyme was performed using ammonium sulphate precipitation across a range of saturation levels. Maximum purity was attained at 90% saturation, followed by dialysis to eliminate residual salts and impurities. Further purification was carried out using Fast protein Liquid Chromatography (FPLC). The molecular mass of the purified protease was determined using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). A consistent band at 57 KDa was observed in both the dialyzed crude extract and the FPLC-purified fraction.

Keywords: *Alcaligenes faecalis*, Fermentation, Skimmed milk, Alkaline protease, FPLC, Dialysis.

1. Introduction

Enzymes play a vital role in the global industrial sector, serving as a highly efficient biocatalystes that accelerate chemical reactions efficiently under mild conditions, significantly lowering energy demands and reducing environmental impact (Singh and Panesar, 2023). Thanks to their precision and versatility, enzymes become the key for improving product quality, enhancing process efficiency and minimizing waste across industries such as food and beverage, textiles, pharmaceuticals, biofeules, and detergents (Allied, Market Research, 2023; Khan, 2025). Enzymes not only boost economic performance but also pave the way for more sustainable and innovative manufacturing practices across diverse applications worldwide.

Proteases (EC 3:4, 11-19, 20-24, 99) are a diverse group of enzymes that play a crucial role in proteolysis - the hydrolysis of peptide bonds in proteins (Watanabe and Hayano, 1994; Singhal *et al*., 2012, and Jisha *et al*., 2013). They are widely distributed across all forms of life, including plants, animals and microorganisms (Polaina and Mac Cabe, 2007). Intracellular bacterial proteases, for instance, are responsible for degrading proteins that are either damaged or functionally obsolete. This targeted breakdown prevents the accumulation of toxic protein aggregates and replenishes the amino acids pool, supporting the synthesis of new proteins and maintaining cellular homoeostasis (Olivares *et al*., 2016). These enzymes are also essential for protein metabolism in human; for instance, they can be used as dietary supplements to enhance protein digestibility (Paulussen *et al*., 2024).

Due to the growing industrial demand for proteases, microbial sources have become economically favorable for large-scale production (Rani *et al*., 2012 and Abdul Razzaq *et al*., 2019). Proteases are generally classified into three main categories: alkaline, acidic and neutral. Additional classifications have also been proposed by Berrett, (1999). These enzymes have numerous industrial applications, as in detergents, leather processing and pharmaceuticals (Singh *et al*., 2001; Shafee *et al*., 2005, and Liu *et al*., 2010).

The genus *Alcaligenes* was first identified in fecal samples due to its presence in the intestines and respiratory tract of vertebrates. Subsequently, members of this genus have been isolated from a wide range of environments, including soil, marine ecosystems, wastewater and other habitats (Rehfuss and Urban, 2005; Tena *et al*., 2014;

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Olusola-Makinde *et al*., 2021; Aullybux *et al*., 2022, and Uttarotai *et al*., 2022). Bacteria of this genus are aerobic, rod-shaped, Gram-negative, and motile due to the presence of flagella (Busse and Stolz, 2006). *Alcaligenes faecalis* is one of the seven species formally recognized in the 9th edition of Bergey's Manual of Determinative Bacteriology (Holt, 1994). Alkaline proteases (EC. 3.4.21-24, 99), in particular, are produced by various bacterial species, the *Alcaligenes faecalis* being a notable producer.

2. Materials and Experimental Methods

2.1. Selection of protease- producing bacteria

2.1.1. Bacterial isolation

Soil samples were aseptically collected in sterile polyethylene bags from various crop-cultivated fields in Giza, Egypt. Under sterile conditions, 5g of each sample was mixed with 100 ml of peptone water (pH 7.2 at 25 ºC) prepared as described by Atlas, (2010). The mixtures were shaken at 150 rpm for 1 hour, then allowed to settle for 30 minutes. Serial dilutions (10^{-1} - 10^{-5}) were prepared. From each dilution, 100 µl was spread onto nutrient agar (NA) plates prepared following Atlas, (2010) and supplemented with nystatin to inhibit fungal growth. Plates were incubated at 37 ºC for 48 hours, and distinct colonies were selected and sub-cultured.

2.1.2. proteolytic activity screening

Selected bacterial isolates were cultured using the inverted agar disc method on skimmed milk agar (SKM-A) prepared according to Atlas, (2010), then plates were incubated. Proteolytic activity was assessed by measuring the diameter of the clear zones around the discs and the growth zones of the isolates.

2.1.3. Assay for protease activity

Under submerged fermentation conditions, 49 ml of autoclaved skimmed milk broth (SKM-B) was dispensed into sterile 250 ml capped bottles, and inoculated with 1 ml of bacterial suspension (1.8 x 10^{-8} CFU) standardized using the 0.5 McFarland method. Cultures were incubated at 37 ºC and 150 rpm for 48 hours in a shaking incubator. Post-incubation, samples were centrifuged at 8000 rpm and 4 ºC for 10 minutes, and the supernatants were collected for protease analysis.

Protease activity was determined using casein as the substrate, following a modified protocol based on Chimbekujwoa *et al*., (2020), incorporating principles from Kunitz, (1946) and Boethling, (1975). The enzyme extract was diluted 1:1 with 0.2 M sodium phosphate buffer (pH 7.0), then 1 ml of this solution was mixed with 2 ml of 1 % casein (prepared in the same buffer) and incubated at 37 ºC for 15 minutes. The reaction was stopped by adding 2.5 ml of 5% trichloroacetic acid (TCA), and the precipitate was removed via Whatman filter paper. For spectroscopic analysis, 1 ml of filterate was mixed with 2.5 ml of 0.5 M sodium carbonate and 1 ml of Folin -Ciocalteau reagent (Alpha Chemika, India), vortexed for 30 seconds, and incubated at 37 ºC for 20 minutes. A blank was prepared by substituting buffer for the enzyme extract, and the standard curve was generated using 5.5 mM tyrosine. Absorbance was measured at 660 nm using a UV-VIS spectrophotometer (photolab 7600 ,WTW), and protease activity was calculated as per Septianingrum *et al*. (2023):

Enzyme activity (unit ml^{-1}) =

$$\frac{\text{tyrosine conc (ppm) x (substrate volume + enzyme volume) (ml)}}{\text{enzyme volume (ml) x incubation time (min)}}$$

Eq. nu. (1)

Under the described assay conditions, one unit of protease activity (U/ml) is defined as the amount of enzyme that releases 1 µg of tyrosine per minute.

2.2. Identification and microscopic examination of isolate

2.2.1. Identification of the selected proteolytic isolate

Molecular identification was performed using 16S rRNA gene sequencing method. DNA was extracted following Macrogene lab protocols, and the 16S rDNA was amplified via PCR using the sequencing primer 785F in conjunction with 907R (Zhang *et al*., 2000). The resulting sequence was compared against the GenBank database, and a phylogenetic tree was constructed using the neighbor- joining method in MEGA11 software.

2.2.2. Light microscopy examination

A Gram-staining bacterial slide was prepared from a 24- hour SKM-A slant culture, following the America Society for Microbiology protocol (Gerhardt *et al.*, 1981) with minor modifications. Sterile distilled water replaced tap water for washing, and crystal violet was applied post-fixation. Morphological features were observed using an Olympus CHBS Laboratory Binocular Microscope (160/0.17) provided with camera.

2.2.3. Scanning Electron Microscopy (SEM)

SEM slides were prepared using a modified method based on Odedina *et al*., (2015). A loopful of 24-hour culture was spread on a clean slide and fixed with 2.5% glutaraldhyde in 50 mM sodium phosphate buffer (pH 7). After 90 minutes, the slide was washed thrice (10 minutes each) with buffer and dehydrated through graded ethanol (30-100%). The dried slide was examined using a FESEM (QUANTA FEG 250).

2.3. Optimization of cultural condition for protease production

2.3.1. Optimization of pH, temperature and incubation time

To evaluate the impact of fermentation condition on protease activity, the isolate was cultured in SKM-B medium under varying pH ranges (3, 5, 5.3, 7, 9 and 11), temperature ranges (4, 20, 37, 40, 50 and 60 ºC) and incubation time ranges (0, 3, 6, 24, 48, 72, 96, 120, 144 and 168 hour). Protease activity was measured post-incubation to identify optimal conditions.

2.3.2. Effect of Carbon and Nitrogen sources

To assess the influence of media composition on enzyme production, the isolate was cultured in SKM-B supplemented with various Carbon sources (glucose, sucrose, maltose, fructose, lactose, carboxymethylcellulose and starch) and Nitrogen sources (yeast extract, urea, ammonium sulphate and sodium nitrate). Protease activity was quantified to determine the most effective nutrient sources.

2.3.3. Comparison of optimized vs. original culturing conditions

Optimal parameters from each test were combined into a single optimum test. Protease activity under these conditions was compared to that of the original setup to evaluate enhancement.

2.4. Extraction and purification of protease

2.4.1. Ammonium sulphate fractionation

Protease was precipitated from the crud extract- obtained under optimized culture conditions- by the salting out method using ammonium sulphate at varying saturation levels (20-100 %), following a slightly modified method based on Wang *et al*., (2022). The solution was stirred at room temperature for complete salt dissolution, then incubated at 4 ^{0}C for 2 hours. Precipitates were recovered by centrifugation (10,000 rpm, 15 minutes, 4 ^{0}C), dried at 45 ^{0}C, weighted, and redissolved in 50 mM tris-HCl buffer (pH 9). Ammonium sulphate saturation was calculated using the equation described by Wingfield, (2016):

$$\textbf{Weight (g)} = \frac{G_{sat}\,(S_2 - S_1)}{1 - (P\,S_2)} \qquad \text{Eq. nu. (2)}$$

Where: G_{sat}: A constant = 515.35

(It is the grams of ammonium sulphate /L of saturated solution at 0 ^{0}C).

S_2: Final saturation (i.e.: the target saturation, for example: a 20% saturation is expressed as 0.2.).

S_1: Initial saturation (= Zero).

P : Density (g/mL) = 0.2722 at 0 ^{0}C

2.4.2. Fractions evaluation

Collected fractions were evaluated through multiple steps, starting with protease activity assessment. Total protein content was measured using the Kjeldahl method (Jamal *et al*., 2020) with a fully automated analyzer equipped with an auto-sampler (BKN-986). Specific protease activity was calculated using Equation number (3) (Mega *et al*., 2020), while the yield percentage and purification fold were determined using Equations (4) and (5) (Islam *et al*., 2009; Arshad *et al*., 2017), respectively as follows :

Specific enzyme activity (unit/mg protein) =

protease activity (unit/ mL) / total protein (mg/mL)

Eq. nu. (3)

% yield=

(activity in the fraction/ activity in crude extract)X 100

Eq. nu. (4)

Purification fold =

specific activity / specific activity of crude extract

Eq. nu. (5)

2.4.3. Dialyses and Anion Exchange Chromatography

The purified protease fraction was dialyzed for 24 hour at room temperature (23-17 ^{0}C ± 1) with gentle stirring (80 rpm). The extract was processed using an ÄKTA Avant 150 FPLC system (GE Healthcare Life Sciences), following a modified protocol based on Ullah *et al*., 2022. Prior to chromatography, the sample was mixed with 50 mM tris-HCl buffer (pH6), and 1ml loaded onto a HiTrap DEAE FF weak anion exchange column. Elution was performed with a linear gradient of sodium chloride (0-1 M) in buffer B at constant flow rate of 1mL /min. Fractions exhibiting absorbance at 280 nm were collected and assayed for protease activity. The fraction displaying the highest enzymatic activity was stored at - 23 ^{0}C for further analysis.

2.4.4. SDS-PAGE analysis

The molecular weight of purified protease that gave the highest activity for both the dialyzed crude extract and FPLC fraction was assessed via SDS-PAGE on a 15% (w/v) polyacrylamide gel, following Laemmli's method (1970) as detailed by Banik *et al.*, (2018). Protein bands were visualized using Coomassie Brilliant Blue staining (Merck, Germany), and molecular weight markers (15-260 KDa) served as references.

2.5. Statistical analyses

Statistical analyses were conducted using IBM SPSS Statistics (version 27). All experiments were performed in triplicate (n=3) to ensure reproducibility and reliability of the results. Protease activity assays were evaluated using one -way analysis of variance (ANOVA). Additionally, comparisons of total protein content, protease activity and specific protease activity across fractions obtained via ammonium sulphate precipitations, and FPLC fractions were analyzed using one- way ANOVA followed by Tukey's Honestly Significant Difference (HSD) post hoc test to determine pairwise differences. Data are presented as mean ± standard deviation (SD). Statistical significance was reported where applicable, and error bars representing SD are included in the corresponding graphical representations.

3. Results and Discussion

Most biochemical reactions within living cells rely on enzymes for their functionality and survival (Pandey, 2024). Among these, proteases are a class of enzymes that catalyze the hydrolysis of proteins by cleaving peptide bonds, thereby generating polypeptides or free amino acids. These enzymes are produced both intracellulary and extracellulary by a wide range of microorganisms (Moo-young and Chisti, 1994, and Gupta *et al*., 2002). In the present study, twenty-three bacterial isolates - previously cultured on nutrient agar (NA)- were evaluated for their ability to produce protease using Skimmed milk agar (SKM-A) medium supplemented with casein as the substrate. Of these, six bacterial isolates demonstrated clear zones surrounding the culture discs, accompanied by visible growth beyond the disc area (Figure 1a & b), indicating proteolytic activity. This approach served as a qualitative assessment of protease production. Subsequently, the protease activity of these six isolates was quantitatively analyzed to identify the isolate producing the highest enzyme concentration (Figure 3).

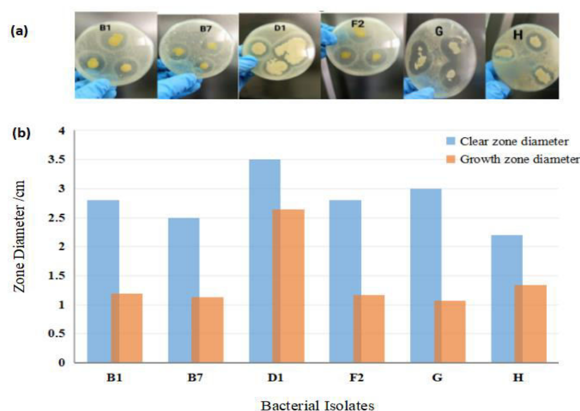

Figure 1. The clear zone and growth zone diameters for the six protease -producing bacterial isolates.

Protease enzymes hydrolyze the protein substrate casein, releasing its constituent amino acid tyrosine, which was quantitatively measured to assess protease activity (Benimana *et al*., 2020). A standard curve for tyrosine concentration was prepared as illustrated in Figure (2). Among the tested isolates, isolate D1 demonstrated the highest level of protease activity (Figure 3). This observation was statistically validated, with a p-value of 0.003 (< 0.05) indicating strong evidence of significant variation in protease activity among the isolates. Furthermore, the F-value (= 6.8) for the test reinforcing the conclusion that the differences observed were not due to random variation but reflect genuine biological differences in enzyme production across the isolates.

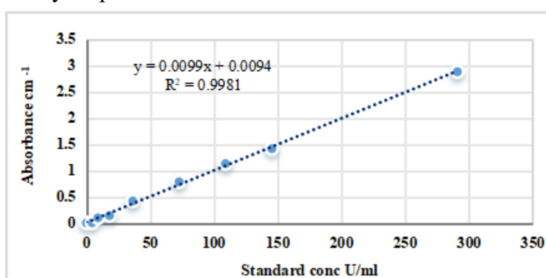

Figure 2. Tyrosine Standard curve.

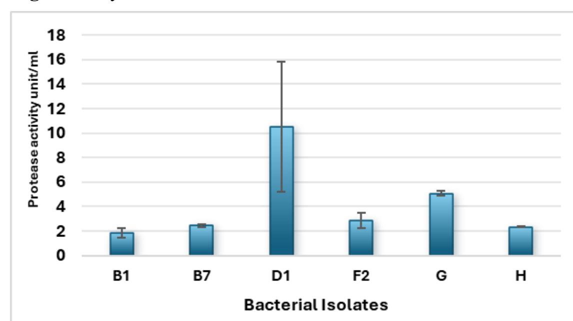

Figure 3. Screening of protease activity for the six protease-producing isolates.

The identification of isolate D1 was confirmed as *Alcaligenes faecalis* through DNA sequencing using the 16s rRNA method. BLAST analysis revealed a 98% sequence identity with *Alcaligenes faecalis* strain P156, indicating a high level of homology. According to Janda and Abbot, (2007), bacterial species identification is considered valid when the 16S rRNA gene sequence similarity is ≥ 97%, supporting the classification of isolate D1 as *Alcaligenes faecalis*. The 16S rDNA sequence of the strain was submitted to the GenBank database under the accession number PQ357195 (EG1-AMY). A phylogenetic tree was subsequently constructed to illustrate its evolutionary relationship with closely related strains (Figure 4).

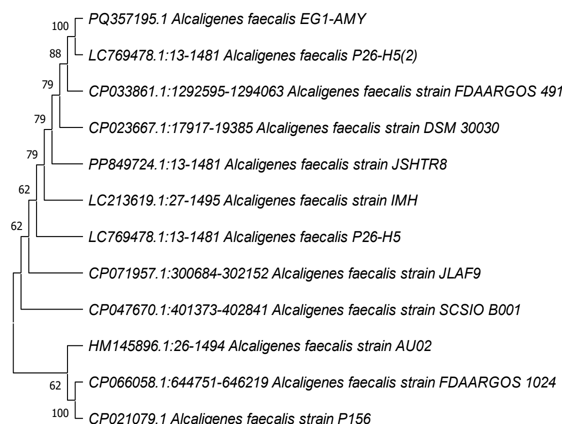

Figure 4. The phylogenetic tree for *Alcaligenes faecalis* PQ357195 (EG1-AMY)

Alcaligenes faecalis is a Gram-negative, rod-shaped bacterium observed to form long chains under scanning electron microscopy (Figure 5b). This species is characterized by its motility, facilitated by peritrichous flagella which were visible under light microscopy (Figure 5a) (Fatima *et al*., 2020; Quach *et al*., 2023). In general, flagellar-driven motility provides bacteria with a significant adaptive advantage, allowing them to actively move toward nutrient -rich environments or sites and steer away from harmful conditions. This locomotion enhance their capacity to locate, colonize, and persist within ecological niches that are support their growth and long-term survival (Creppy *et al*., 2019; Makarchuk *et al*., 2019; Colin *et al*., 2021, and Zheng *et al*., 2021). Such motility may explain the wide growth zone observed for the strain (isolate D1) as presented in Figures 1a and 1b.

Motile bacterial cells generally outperform non-motile ones in liquid media due to their ability to navigate the environment using flagella. Initially, *Alcaligenes faecalis* (isolate D1) was incubated in a shaking incubator to enhance aeration and facilitate uniform nutrient distribution throughout the medium. However, this method was later discontinued, and the cultures were subsequently maintained in a static incubator to better suit the growth characteristics of the strain.

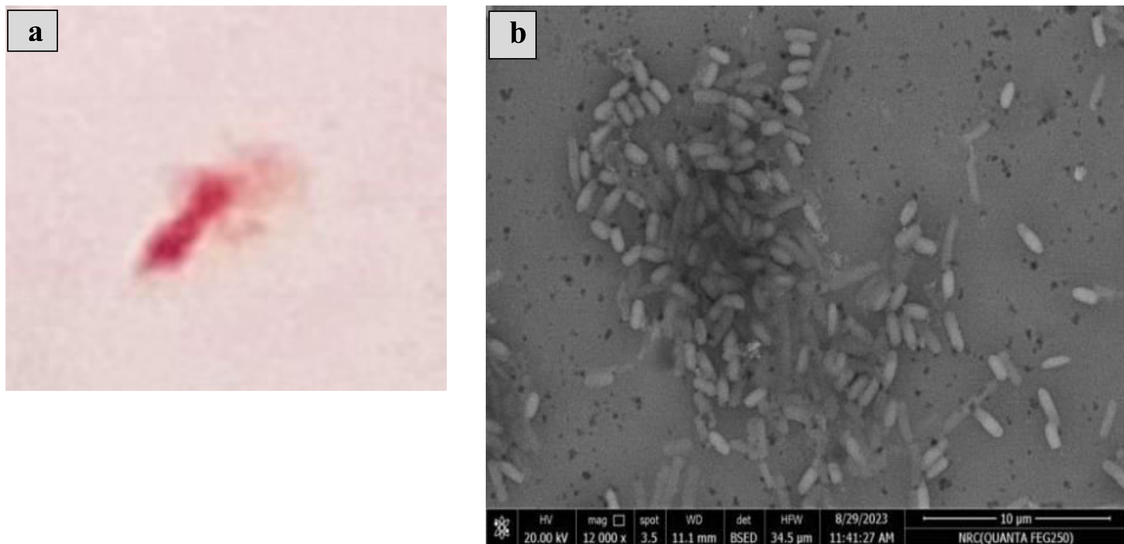

Figure 5. Examination of *Alcaligenes faecalis* PQ357195 (EG1-AMY) under light microscopy (a) and scanning electron microscopy (b).

In this study, the cultivation conditions for *Alcaligenes faecalis* and its protease production were evaluated under various selected parameters. The impact of these parameters was assessed by measuring enzyme activity. Maximum protease activity was recorded at pH 9, indicating that the isolate performs best under alkaline conditions. However, enzyme activity declined sharply at pH 11, which exceeds the optimal range (Figure 6a). These findings align with previous reports identifying *A. faecalis* as a potent producer of alkaline protease (Marathe *et al.*, 2018 and Obichi and Ezebuiro, 2021). Temperature optimization experiments were conducted across a range of 4-60 ^{o}C (Figure 6b). The highest protease activity was observed at 37 ^{o}C, which corresponds to the original culturing temperature. This result is consistent with findings by Annamalai *et al*., (2011). Regarding incubation time, 96 hours yielded the highest protease activity (Figure 6c). Prolonged incubation beyond this point led to a notable decline in enzyme activity. Interestingly, this result contrasts with several studies that reported optimal activity at 72 hour (Srividya, 2011 and Khatoon *et al.*, 2023). *Alcaligenes* species are chemoorganotrophic bacteria capable of utilizing a broad spectrum of carbon sources (Robinson, 2014). To assess the influence of carbon sources, glucose in the original medium was substituted with various alternatives. All tested carbon sources supported bacterial growth, but glucose resulted in the highest protease activity (Figure 6d). Similarly, when nitrogen sources were varied, yeast extract produced the highest enzyme activity (Figure 6e). These outcomes are consistent with previous findings by Marathe *et al*., (2018) on alkaline protease produced by *A. faecalis*. The statistical analysis confirmed that all tested parameters had a significant impact on protease production by *A. faecalis*. Variations in pH ($F = 4.1$, $p = 0.022$), temperature ($F = 23.1$, $p < 0.001$), incubation time ($F = 8.9$, $p < 0.001$), carbon source ($F = 12.5$, $p < 0.001$), and nitrogen source ($F = 16.8$, $p < 0.001$) each produced statistically significant differences in protease activity. These results underscore the importance of optimizing environmental and nutritional factors to enhance protease yield.

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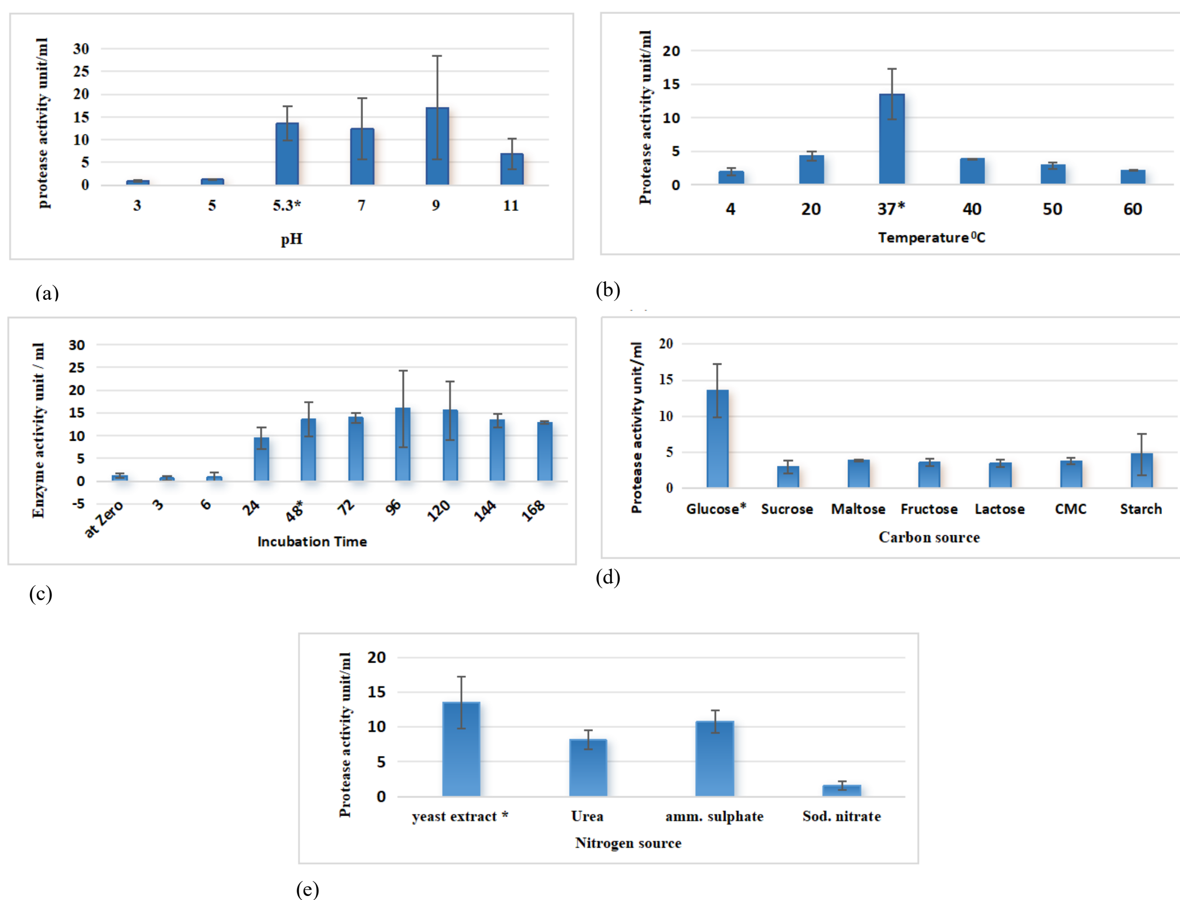


* : indicates the used parameter in the original test.

Figure (6): Optimization of cultivation conditions for protease production by *Alcaligenes* faecalis PQ357195 (EG1-AMY).

The combined impact of all optimized parameters were assessed by applying them in a single test and comparing the results to the original conditions. As illustrated in Figure 7, the optimized setup led to marked increase in protease activity, confirming the effectiveness of the optimization. The statistical analysis yielded an F-value of 55 and a p-value of 0.002, indicating a strong and reliable improvement in protease yield due to the applied optimization strategy.

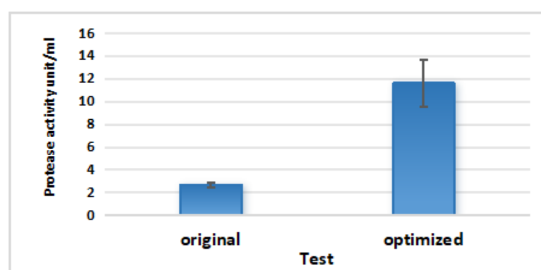


Figure 7. Comparative analysis of protease yield under original and optimized cultivation conditions in *Alcaligenes faecalis* PQ357195 (EG1-AMY).

The crude extract containing the extracellular alkaline protease was successfully subjected to successive purification steps. The initial partial purification was achieved by using the salting- out method at varying ammonium sulphate saturation levels (Table 1). Protein precipitation using ammonium sulphate had a statistically significant impact on total protein concentration ($F = 8667.0$ and $p < 0.001$), protease activity ($F = 548.8$ and $p < 0.001$) and enzyme specific activity ($F = 171.4$ and $p < 0.001$). These effects were highly significant ($p < 0.05$), as further evidenced by the high F - statistic values, which indicate substantial between- group differences. The highest protease recovery was achieved at 90 % saturation as presented in Table 1. The ammonium sulphate precipitation method relies on the principle that the ions of the salt compete for water molecules, thereby reducing protein solubility. This reduction promotes the interaction among the proteins molecules resulting their aggregation and subsequent precipitation (Vetal & Rathod, 2014 and Urgessa *et al.*, 2019). Although 100% saturation is theoretically intended to precipitate all proteins, it may induce protein unfolding and subsequent enzymatic inactivation. This is evidenced by the observed decline in protease activity, specific activity, yield and purification fold compared to 90% saturation as shown in Table 1. The observed variations in specific protease activity across different saturation levels likely reflect the heterogeneous distribution of enzymatic and non-enzymatic proteins as well as protease inhibitors, each exhibiting different solubility profiles at varying salt concentrations (Scopes, 1994; Meza-Espinoza *et al.,* 2018; Khadka *et al*., 2024).

Table 1: Summary of alkaline protease fractions obtained via precipitation by ammonium sulphate.

Ssaturation (%)	Ammonium sulphate (g/mL)	Precipitate (g)	Total protein (mg/mL)	Protease activity (unit/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
crude	-	-	14.0 ± 0.12	11.6 ± 0.03	0.83 ± 0.006	-	-
20%	10.9	0.25	4.70 ± 0.03 [a]	0.03 ± 0.05	0.01 ± 0.011	0.8	0
30%	16.8	1.58	4.80 ± 0.0 [a]	0.50 ± 0.06 [d]	0.11 ± 0.012	4.3	0.1
40%	23.1	1.84	5.50 ± 0.03	1.36 ± 0.34 [d]	0.25 ± 0.060 [c]	11.7	0.3
50%	29.8	1.98	6.00 ± 0.1	1.58 ± 0.09 [d]	0.26 ± 0.011 [c]	13.6	0.3
60%	38.8	2.41	7.00 ± 0.09	2.82 ± 0.17 [c]	0.40 ± 0.029 [a]	24.3	0.5
70%	44.5	2.67	7.70 ± 0.03	3.46 ± 0.28 [b,c]	0.45 ± 0.034 [a]	29.8	0.5
80%	52.6	3.28	11.4 ± 0.01	4.32 ± 0.03 [a,b]	0.38 ± 0.002 [a,b]	37.3	0.5
90%	61.3	5.14	12.4 ± 0.1	4.97 ± 0.54 [a]	0.40 ± 0.047 [a]	42.8	0.5
100%	70.6	9.97	13.5 ± 0.05	4.73 ± 0.29 [a]	0.35 ± 0.02 [a,b]	40.7	0.4

The protein precipitated at 90% ammonium sulphate saturation was dialyzed to remove residual salts and impurities, followed by further purification using FPLC. Based on the absorbance at optical density 280 nm, seven distinct fractions were collected (Figure 8) and subsequently assayed for protease activity. Among these, the 1C4 fraction exhibited the highest protease activity (Figure 9). Notably the overall enzyme yield following the purification was relatively low, likely attributed to protease autolysis occurring during the successive purification steps (EL-Eskafy *et al*., 2016). This may account for the observed variation in protease activity across the FPLC fractions, showing a marginally significant difference (p=0.055) suggestive of potential fraction-specific enrichment. The corresponding F-value (F=2.8) indicates that the observed differences among the fractions are meaningful but not pronounced.

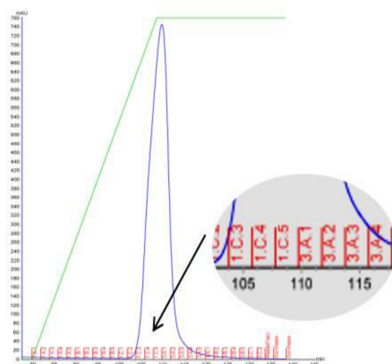


Figure 8: Elution profile from FPLC at optical density 280 nm.

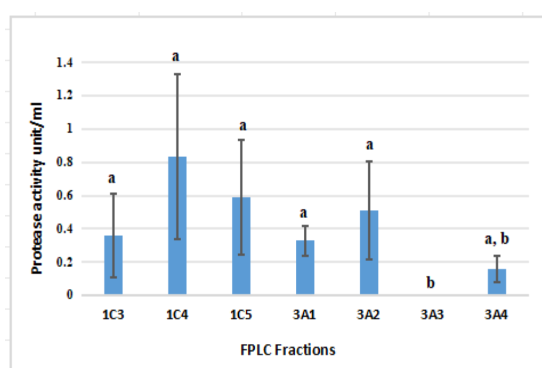
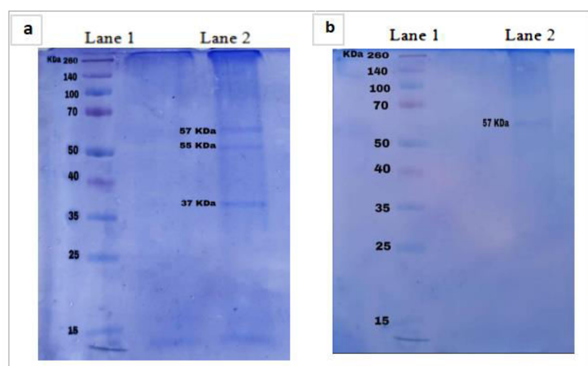


Figure 9: Evaluation of protease activity in the FPLC fractionations.

Alkaline proteases purified from various bacterial species exhibit considerable wide range of molecular mass (Hashmi *et al.,* 2022; Ullah *et al.,* 2022 ; Salim *et al*., 2023; Santos *et al*., 2024 ; Punnarath *et al.,* 2025). In this study, SDS-PAGE analysis of the dialyzed crude protease extract obtained at 90% ammonium sulphate saturation revealed three bands at approximately 37, 55 and 57 KDa as shown in Figure 10a. Subsequent purification using FPLC confirmed the enzyme's purity, as evidenced by a single band at approximately 57 KDa, corresponding to its molecular mass as appeared in Figure 10b.



Lane 1 : the marker molecular weight and Lane 2 :The eluted fraction

Figure 10: SDS-PAGE analysis of dialyzed crude protease extract and FPLC- purified 1C4 fraction.

4. Conclusion

This study aimed to isolate bacterial strain capable of producing protease with high concentration and activity. Among the isolates, the isolate D1 - later identified as *Alcaligenes faecalis* and registered in GenBank under accession nu. PQ357195 (EG1-AMY) - exhibited the most prominent clear zone and growth zone diameters, indicating superior proteolytic potential.

Following a series of optimization experiments, all optimized parameters were combined and applied in a single test. Protease production was markedly improved under conditions utilizing glucose and yeast extract as carbon and nitrogen sources, respectively, at pH 9 and incubated at 37 ^{o}C for 96 hours. Compared with the original conditions, this approach significantly enhanced protease production. The enzyme produced was classified as an alkaline protease. The crude extract, precipitated at 90% ammonium sulphate saturation, was subsequently purified using FPLC. SDS-PAGE analysis revealed that the purified alkaline protease had a molecular mass of approximately 57 KDa.

References

Allied, Market Research. 2023. Global Enzyme market, Opportunity and Forecast, 2018-2024, [cited 2023 on 6-29-2023]; Available from: www.alliedmarketresearch.com/enzymes-market

Atlas RM. 2010. Handbook of microbiological media, Fourth Edition. CRC Press, Taylor and Francis Group.

Annamalai N, Kumar A, Saravanakumar A, Vijaylakshmi S and Balasubramanian T. 2011. Characterization of protease from *Alcaligenes faecalis* and its antibacterial activity on fish pathogens. *J of Environ Biol.,* 32:781-786.

Arshad ZIM, Amid A, Yusof F, Sulaiman SZ, Mudalip SKA, Man RC and Shaarani SM-d. 2017. Comparison of purification methods to purify recombinant bromelain from *Escherichia coli* BL21-A1. *Malaysian J Analyt Sci.*, 21 (4): 958 - 971.

Aullybux AA, Puchooa D, Bahorun T, Jeewon R, Wen X and Matin P. 2022. Antioxidant and cytotoxic activities of exopolysaccharides from *Alcaligenes faecalis* species isolated from the marine environment of mauritius. *J Poly Environ*., 30 (4): 1462–1477.

Banik S, Biswas S and Karmakar S. 2018. Extraction, purification, and activity of protease from the leaves of *Moringa oleifera*. *F1000Res.,* 7: 1151.

Barrett AJ. 1999. Peptidases: a view of classification and nomenclature. In: Turk V. (Ed), **Proteases: New Perspectives**. Springer Basel AG.

Benimana F, Potoroko IY, Bagale DrU, Olivier M and Felicien K. 2020. Protease activity in flesh leaves of *Bidens pilosa*. *Int J Sci Technol Res*., 9 (3).

Boethling RS. 1975. Regulation of Extracellular Protease Secretion in *Pseudomonas maltophilia.* *Ameri Soc Microbiol*., 123 (3): 954-961.

Busse HJ and Stolz A. 2006. *Achromobacter*, *Alcaligenes* and Related Genera. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H and Stackebrandt E. (Eds), **The Prokaryotes : A Handbook on the Biology of Bacteria,** 3rd edn, volume 5: Proteobacteria: Alpha and Beta Subclasses. Sringer Science+Business Media, LLC. Chapter 3.2.4:675-700

Chimbekujwoa KI, Ja'afarua MI and Adeyemo OM. 2020. Purification, characterization and optimization conditions of protease produced by *Aspergillus brasiliensis* strain BCW2. *Sci Afr*, 88.

Colin R, Ni B, Laganenka L and Sourjik V. 2021. Multiple functions of flagellar motility and chemotaxis in bacterial physiology. *FEMS Microbiol Rev*., 45(6).

Creppy A, Clément E, Douarche C, D'Angelo MV and Auradou H. 2019. Effect of motility on the transport of bacteria populations through a porous medium. *Ameri Phys Soc.- Phys rev flu.*, 4 (013102).

EL-Eskafy HE, Abdel-Hamid MS, Abbas RN, Hamza HA and El-Zanaty AH. 2016. Production of Thermostable Protease From *Bacillus amyloliquefaciens* :An Egyptian Marine Isolate. *J Agri Chem Biotechnol.* -Mansoura Univ., 7(8): 227 - 234.

Fatima T, Mishra I, Verma R and Arora NK. 2020. Mechanisms of halotolerant plant growth promoting *Alcaligenes* sp. involved in salt tolerance and enhancement of the growth of rice under salinity stress. *3 Biotech*, 10(8):361.

Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR and Phillips GB. 1981. Manual of Methods for General Bacteriology. Über die isoliere Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. *Fortsch der Med*., 2:185-189. ASM Press, Washington D.C. Gram. C.

Gupta R, Beg QK and Lorenz P. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol.,* 59.

Hashmi S, Iqbal S, Ahmed I, Janjua HA. 2022. Production, optimization, and partial purification of alkali-thermotolerant proteases from newly isolated *Bacillus subtilis* S1 and *Bacillus amyloliquefaciens* KSM12. *Processes*; 10 (1050).

Holt JG. 1994. Bergey's manual of determinative bacteriology- 9th ed. Lippincott Williams and Wilkins, Baltimore.

Islam MA, Parveen F, Hossain K, Khatun S, Karim MdR, Kim GS, Absar N and Haque MdS. 2009. Purification and biochemical characterization of lipase from the dorsal part of Cirrhinus reba. *Thai J Agri Sci.*, 42 (2): 71-80.

Jamal SD, Jamil DM and Khidhir ZK. 2020. Protein determination in some animal products from Sulaymaniyah markets using Kjeldahl procedure. *J Food and Dairy Sci.*- Mansoura Univ.; 11 (12): 343 - 346.

Janda JM and Abbot SLS. 2007. Gene sequencing for bacterial identification in the diagnostic laboratory. *J Clini Microbiol.*, 45: 2761-2764.

Jisha VN, Smitha RB, Pradeep S, Sreedevi S, Unni KN, Sajith S, Priji P, Josh MS, and Benjamin S. 2013. Versatility of microbial proteases. *Adv Enz Res*., 1: 39-51.

Khadka DB, Pahadi T, Aryal S, Karki DB. 2024. Partial purification and characterization of protease extracted from kinema. *Heliyon*, 2024:27173.

Khan MF. 2025. Recent Advances in Microbial Enzyme Applications for Sustainable Textile Processing and Waste Management. *Science*, 7(46).

Khatoon N, Ullah N, Sarwar A, Ur Rahman S, Khan AA, Aziz T, Alharbi M and Alshammari A. 2023. Isolation and identification of protease producing *Bacillus* strain from cold climate soil and optimization of its production by applying different fermentation conditions. *App eco environ resear*., 21(4): 3391-3401.

Kunitz M. 1946. Crystalline soybean trypsin inhibitor: II. General properties. *J General Physiol*.: 291-310. From the Laboratories of The Rockefeller Institute/or Medial Reaeamk, Princeton, New Jersey.

Laemmli UK. 1970. Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature*, 227: 680.

Liu J, Huang XF, Lu LJ, Xu JC, Wen Y, Yang DH and Zhou Q. 2010. Optimization of biodemulsifier production from *Alcaligenes* sp. S-XJ-1 and its application in breaking crude oil emulsion. *J Hazar Mater*, 83 (1–3) : 466–473.

Makarchuk S, Braz VC, Araújo NAM, Ciric L and Volp G. 2019. Enhanced propagation of motile bacteria on surfaces due to forward scattering. *Nature communic*., 10 (4110).

Marathe SK, Vashistht MA, Prashanth A, Parveen N, Chakrabort S and Nair SS. 2018. Isolation, partial purification, biochemical characterization and detergent compatibility of alkaline protease produced by *Bacillus subtilis*, *Alcaligenes faecalis* and *Pseudomonas aeruginosa* obtained from sea water Samples. *J Gen Eng Biotechnol*., 16: 39–46.

Mega O, Sumantri C, Arief II and Budiman C. 2020. Purification and proteolytic activity of caseinolytic protease (Clp) from *Lactobacillus plantarum* IIA-1AS. AIP conference proceedings, 2296, 020046.

International conference on science and applied science (ICSAS2020), Surakarta, Indonesia.

Meza-Espinoza L, Vivar-Vera Mde-LA, Garcı´a-Magan˜a Mde-L, Sa´yago-Ayerdi SG, Chaco´n-Lo´pez A, Becerrea-Verdı´n EM and Montalvo-Gonza´lez E. 2018. Enzyme activity and partial characterization of proteases obtained from *Bromelia karatas* fruit and compared with *Bromelia pinguin* proteases. *Food Sci Biotechnol*., 27(2):509–517.

Moo-young M and Chisti Y. 1994. Biochemical engineering in biotechnology. *Pure and App Chem.,* 66 :117–136.

Obichi EA and Ezebuiro V. 2021. Effects of temperature and pH on the stability of protease produced by *Alcaligenes faecalis* P2. J *Appl Life Sci Internat.*, 24 (11): 1-9.

Odedina GF, Vongkamjan K and Voravuthikunchai SP. 2015. Potential Bio-Control Agent from *Rhodomyrtus tomentosa* against *Listeria monocytogenes*. *Nutrients*, 7: 7451-7468.

Olivares AO, Baker TA, Sauer RT. 2016. Mechanistic insights into bacterial AAA+ proteases and protein remodelling machines. *Nature Rev Microbiol.*, 14(1): 33–44.

Olusola-Makinde OO, Arotupin DJ and Okoh AI. 2021. Optimization of haemolysin production by *Alcaligenes* species isolated from abattoir wastewater samples in Akure, Ondo State, Nigeria. *Kuw J Sci*., 49 (3): 1–19.

Pandey G. 2024. Biochemical Reactions: The Chemical Basis of Life. *J Med Medic Sci*., 15 (6):1-2.

Paulussen KJM, Askow AT, Deutz MT, McKenna CF, Garvey SM, Guice JL, Kesler RM, Barnes TM, Tinker KM, Paluska SA, Ulanov AV, Bauer LL, Dilger RN and Burd NA. 2024. Acute Microbial Protease Supplementation Increases Net Postprandial Plasma Amino Acid Concentrations After Pea Protein Ingestion in Healthy Adults: A Randomized, Double-Blind, Placebo-Controlled Trial. *J Nutrition*, 154 : 1549–1560

Polaina J and MacCabe AP. 2007. Industrial enzymes; structure, function, and applications. Dordrecht: Springer.

Punnarath NP, Meppat A, Subash A, Ponraj SW and Arunachalam S. 2025. Screening and characterization of thermostable protease-producing bacteria isolated from slaughter site soil samples from Southern India. *J App Biol Biotechnol*., 13(1):159-168.

Quach NT, Loan TT, An Nguyen TT, Nguyen Vu TH, Pham QA, Chu HH, Phi Q-T and Thuoc DV. 2023. Phenotypic and genomic characterization provide new insights into adaptation to environmental stressors and biotechnological relevance of mangrove *Alcaligenes faecalis* D334. *Res Microbiol.*, 174:103994.

Rani K, Rana R and Datt S. 2012. Review on latest overview of Protease. *Inter J Curr Life Sci.,* 2 (1):12.

Abdul Razzaq, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A and Ashraf M. 2019. Microbial protease applications. *Front Bioeng Biotechnol*., 7 (110).

Rehfuss M and Urban J. 2005. *Alcaligenes faecalis* subsp. *phenolicus* subsp. nov. a phenol-degrading, denitrifying bacterium isolated from a graywater bioprocessor. *System Appl Microbio.,* 28 (5): 421–429.

Robinson RK. 2014. Encyclopedia of food microbiology, 2nd edn. New Yourk (NY): Academic press.

Salim MSM, Mohameda SS, Abdelhamida SA and Hamed AA. 2023. Characterization and Purification of Alkaline Protease from Novel *Bacillus subtilus* MS for Detergent Additives. *Egyp J Chem*., 66 (3): 281 – 288.

Santos MPF, de Souza Junior EC, Villadóniga C, Vallés D, Castro-Sowinski S, Bonomo RCF and Veloso CM. 2024. Proteases: importance, immobilization protocols, potential of activated carbon as support, and the importance of modifying supports for immobilization. *J Biotechnol*., 13 (13).

Scopes R.K. 1994. Separation by precipitation. In: **Protein purification: principles and practice**. Springer, New York, NY, pp. 71–101,

Septianingrum WR, Sanjaya EH, Alvionita M and Suharti S. (2023). Optimization of arotease activity by *Bacillus haynesii* BK1H using response surface methodology. E3S Web of Conferences, 481, 05002. International Conference on Sustainable Chemistry (ICSChem 2023), section of Green Chemistry.

Shafee N, Aris SN, Rahman RZA, Basri M and Salleh AB. 2005. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus s*train 146. *J Appl Sci Res*, 1: 1-8.

Singh J and Panesar PS. 2023. Industrial enzymes: Basic information, assay, and applications (Chapter 20). In: **Basic Biotechniques for Bioprocess and Bioentrepreneurship**, academic press, pp. 295-309.

Singh J, Batra N and Sobti RC. 2001. Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. *Proc Biochem*., 36 : 781.

Singhal P, Nigam VK and Vidyarthi AS. 2012. Studies on production, characterization and applications of microbial alkaline protease. *Inter J Adv Biotechnol Res*, 3 (3): 653-669.

Srividya S. 2011. Influence of process parameters on the production of detergent compatible alkaline protease by a newly isolated *Bacillus* sp. Y. *Turk J Biol*., 35 (2):177-182.

Tena D, Fernández C and Lago MR. 2014. *Alcaligenes faecalis*: an unusual cause of skin and soft tissue infection. *Japan J Infec Dis*., 2014 :2164.

Ullah N, Ur Rehman M, Sarwar A, Nadeem M, Nelofer R, Shakir HA, Irfan M, Idrees M, Naz S, Nabi G, Shah S, Aziz T, Alharbi M, Alshammari A and Alqahtani F. 2022. Purification, characterization, and application of alkaline protease enzyme from a locally isolated *Bacillus cereus* strain. *Ferm.,*8: 628.

Urgessa OE, Itana DD and Raga TO. 2019. Extraction of papain from papaya (*Carica papaya* L.) fruit latex and its application in transforming tannery raw trimming. *Eth J Sci Sustain Dev*., 6 (2).

Uttarotai T, Sutheeworapong S, Crombie AT, Murrell JC, Mhuantong W, Noirungsee N, Wangkarn S, Bovonsombut SJ, McGenity T and Chitov T. 2022. Genome characterisation of an isoprene-degrading *Alcaligenes* sp. isolated from a tropical restored forest. *Biol.*, 11(4): 519.

Vetal MD and Rathod VK. 2014. Three phase partitioning a novel technique for purification of peroxidase from orange peels (*Citrus sinenses*). *Food bio-prod process*., 94: 284–289.

Wang H, Xu J, Kong B, Liu Q, Xia X and Sun F. 2022. Purification and characterization of the protease from *Staphylococcus xylosus* A2 isolated from harbin dry Sausages. *Foods*, 11: 1094.

Watanabe K and Hayano K. 1994. Estimate of the source of soil protease in upland fields. *Biol Ferti Soils*, 18: 341-346.

Wingfield PT 2016. Protein precipitation using ammonium sulfate. *Curr Protoc Protein Sci*.- author manuscript; available in PMC 2016 April 01.

Zheng S, Bawazir M, Dhall A, Kim HE, He L, Heo J and Hwang G. 2021. Implication of surface properties, bacterial motility, and hydrodynamic conditions on bacterial surface sensing and their initial adhesion. *Fron Bioeng Biotechnol*., 9: 643722.

Zhang Z, Schwartz S, Wagner L and Miller W. 2000. A greedy algorithm for aligning DNA sequences. *J comput biol.,* 7 (1-2): 203-214.