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Isolation, screening, and characterization of protease-producing bacteria from the proteinous waste-dumped soil

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Abstract

Proteases are ubiquitous enzymes found in all living organisms and have versatile applications across industrial, environmental, medical, and biotechnological fields. Bacterial proteases are extracellular and easier for downstream processing. This study aimed to identify effective protease-producing bacteria from soil contaminated with protein-rich waste. The study revealed 17 protease-producing bacteria from soils contaminated with dairy, poultry, and cafeteria waste. Of these, 11 (64.7%) isolates showed a significant zone of inhibition on skim milk agar, indicating their potential for enzyme production. The primary screening identified five bacterial isolates with strong protein-degrading capability (24.3–39.7 mm) within 72 hours of incubation. Three of which (PWS-2, MWS-5, and PWS-4) were further tested and found to produce significant amounts of protease (180U/mL, 172U/mL, and 160U/mL). These isolates were gram-positive bacteria. PWS-2 and PWS-4 are rod-shaped, while MWS-5 is a coccoid bacterium, sourced from poultry and dairy waste soils, respectively. They grew in a wide range of temperatures (5–40°C), pH levels (4–9), and salt concentrations (0.2–6%), with substantial biomass production. This indicates that soil contaminated with protein waste is a promising source of proteolytic enzyme-producing bacteria.

Keywords: Bacteria, zone of inhibition, proteinous waste, protease production

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1. Introduction

Protease is a universal hydrolytic enzyme produced by all higher and microscopic organisms, including biological entities (viruses) living on the earth. It catalyzes different types of protein molecules and is used to execute their physiological functions in living things. It is a highly applicable enzyme in food, feed, dairy, leather, textile, detergent, and pharmaceutical processing industries, in which its mass production has been highly demanding (Nigam, 2013).

Microscopic organisms, including fungi, are key protease-producing entities found abundantly in soil, water, food, and animal guts, as well as in extreme environments like waste dumping sites, rocks, glaciers, hot springs, and deep-sea vents (Hamza and Azmach, 2018; Masi et al., 2021; Raut et al., 2012). These organisms can be isolated and mass-produced using artificial media if conditions are suitable for their growth (Sani and Dahman, 2010). In particular, bacteria are fast-growing and efficient producers of extracellular proteases that are directly discharged into the fermentation medium, making downstream processing easier (Hamza and Azmach, 2018; Kasana et al., 2011; Nigam, 2013).

Bacterial proteases are among the most fascinating, versatile, and valuable for mass production in various industries. It can be stored under preservative conditions for long periods without significant loss or alteration of its catalytic activities (Perry et al., 2025). Reports revealed that roughly 60% of the world's total industrial enzyme sales are accounted for by products derived from different bacterial strains(Temam, 2017).

Thus, various types of protease-producing bacterial strains have been isolated from different sources for use in industrial, environmental, and biotechnological applications (Kumar and Dwivedi, 2011). Bacterial proteases are effective at removing proteinaceous waste and are highly important in dairy processing and poultry waste management (Ariaeenejad et al., 2022). Their versatile applications in detergent production, food processing, leather processing, pharmaceutical manufacturing, and diagnostics have increased the demand for proteases worldwide (Song et al., 2023).

However, the catalytic efficiency can be significantly determined by various factors such as temperature, pH, salt concentration, and the composition and types of available nutrients (Singh et al., 2014). Several industries, for example, operate under extreme conditions like very low or high temperatures, acidic or alkaline environments, varying salt concentrations, and different nutrient compositions, all of which influence the efficiency of biological catalytic activities of the enzyme(Abebe et al., 2014).

Soil contaminated with proteinaceous waste is a nutrient-rich, selective ecological niche that continuously pressures resident microorganisms to produce extracellular proteases for survival and competition. Despite its high potential as a reservoir of robust, efficient protease-producing bacteria, this environment remains comparatively underexplored, particularly in identifying strains capable of producing high levels of extracellular protease and tolerating adverse conditions.

Given the increasing global demand for proteases that tolerate adverse conditions in the food, detergent, leather, pharmaceutical, and waste management industries, there remains a continued need to isolate and characterize bacterial strains that combine high protease productivity with environmental resilience (Vinothini et al., 2025). Therefore, isolating and screening bacterial strains that are tolerant to adverse conditions and produce high levels of protease from different sources is an ongoing task to obtain effective and catalytically active enzymes. The main objective of this study was to isolate and characterize protease-producing bacteria from soil contaminated with proteinaceous waste and to screen for efficient strains for mass production.

2. Materials and Methods

2.1.Sample collection

A total of 15 composite proteinaceous waste soil samples were collected from the waste dumping sites of the students' cafeteria (5 samples), Menkorere Agro-industrial Dairy Production Center (5 samples), and poultry (5 samples) at Debre Markos University. Soil samples from each site were obtained using a soil auger from a depth of 10 to 15 cm in triplicate. A 200 g composite soil sample from each site was placed in polyethylene bags sterilized with 70% ethanol and transported to the microbiological laboratory at the Department of Biotechnology, Debre Markos University, for bacterial isolation.

2.2.Isolation of bacteria

The 10 gof soil was suspended in 90 mL of sterilized normal saline solution in a 150 mL conical flask. The soil suspension was homogenized by manual agitation for 20 sec and then serially diluted tenfold using normal saline solution. Next, 100 µL of the suspension from each of the 10^{-4}, 10^{-5}, and 10^{-6} dilution factors was spread onto autoclaved (121°C) nutrient agar medium prepared in Petri dishes and incubated at 37°C for 48 hours (Masi et al., 2021). The bacterial isolates with different colony features were separately transferred onto fresh nutrient agar medium for purification. The purified isolates were maintained in agar slants at 4°C for further work.

2.3. Preliminary screening of protease-producing bacteria

The skim milk powder-supplemented medium composed of yeast extract (1g/l), skim milk powder (10g/l), NaCl (4g/l), peptone (0.1g/l), and agar (15 g/l) was prepared in a Petri dish (Masi et al., 2021). The medium was spot inoculated with a pure culture of each bacterial isolate in triplicate using an autoclaved toothpick and incubated at 37 ^{0}C. The clear zone-forming bacterial colonies were screened after 24, 48, and 72 hours of incubation. The isolates producing a considerable zone of inhibition around the colony were selected for the second round of screening.

2.4. Rescreening of protease-producing bacteria

2.4.1. Enzyme extraction

The bacterial isolates producing the zone of inhibition were subjected to a rescreening process. They were inoculated into nutrient broth medium supplemented with 5% skim milk powder and incubated at 37 °C for 48 hours. After 48 hours of incubation, 3 mL of suspension was transferred into a 5 mL Eppendorf tube and centrifuged at 10,000 rpm for 10 min. The supernatant was filtered using a syringe fitted with a 0.02 µm pore size filter membrane. The cell-free crude enzyme was used to evaluate casein inhibition and the protease assay (Bhowmik et al., 2015).

2.4.2. Qualitative evaluation of the enzyme

The skim milk powder supplemented agar medium was prepared as presented above and punched aseptically with a sterile cork borer to obtain 6 mm diameter wells. The wells were loaded with 75 µL of crude enzyme and kept in an incubator at 37 °C for 24 hours. The size of the zone of inhibition formed was measured in millimeters after 24 hours of incubation (Bhowmik et al., 2015).

2.4.3. Enzyme assay

The proteolytic activity of crude enzyme was determined using casein as substrate and spectrometric observation at a wavelength of 660 nm. The tyrosine standard curve was obtained from the absorbance of the tyrosine concentrations (10, 20, 30, 40,50, and 60 µg) to determine the protease activity(Masi et al., 2021).

2.5. Cultural characterization ofbacteria

2.5.1. **Colony Morphology**

The shape, color, size, edge, and texture of the selected protease-producing bacterial colonies were evaluated after 24 hours of growth on the nutrient agar medium (Messi et al., 2014).

2.5.2. Gram staining and cellular characteristics

The protease-producing bacterial isolates were Gram-stained by taking a loop full of 24-hour-old culture to a glass slide, heat fixed, flooded with crystal violet (primary stain) for 30 sec, and rinsed with water. The slides were mordant dyed with iodine for 1 min, decolorized with 95% alcohol for 1 min, and counterstained with safranin for 30 sec and subsequently washed with sterile water. The slides were air-dried and observed under a light microscope to distinguish the Gram reaction category, shape of the cell, and cell arrangement (Moyes et al., 2009).

2.6.Biomass production of bacteria

The biomass production of the selected protease-producing bacterial isolates was evaluated by growing in nutrient broth medium for 24 hours at 37 °C, centrifuging at 5000 rpm for 10 min, and the pellet from the bottom of the test tube was measured using a sensitive balance in mg (Masi et al., 2021).

2.7.Physicochemical characterization of bacteria

The bacterial ability to grow under low and high temperature ranges was monitored at 5, 10, 15, 20, 25, 30, 35, 40, and 45 °C (Borge et al., 2001), salinity tolerance at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5,5.5, and 6% (w/v) of NaCl concentration (El-Hassayeb and Aziz, 2016) and the pH range at 4, 4.5, 5, 5.5, 8, and 9 (Singh et al., 2014).

2.8.Data analysis

The data were subjected to one-way ANOVA using SPSS software version 25 to determine the significance level among the isolates' zone of inhibition. Mean separation of zone of inhibition formation was calculated using Tukey's Least Significant Difference (LSD) test at $\rho \leq 0.05$.

3. Result and Discussion

3.1.Isolation of protease-producing bacteria

This study aimed to isolate, screen, and characterize bacteria that produce proteolytic enzymes from various soil samples collected at waste-dumping sites of the students' cafeteria, poultry, and dairy center. The bacteria were isolated from the soil using serial dilution and spread plate methods on nutrient agar medium. Based on the morphological features of bacterial colonies, 160 different isolates were obtained and purified through subsequent sub-culturing.

The highest number of isolates (64) was recovered from dairy waste soil, followed by 56 (35%) from poultry waste soil, and 40 (25%) from cafeteria waste soil (data not shown). This is a significant number compared to the 28 bacterial isolates reported from effluent samples collected from the Modjo leather industry, Ethiopia (Masi et al., 2021).

Table1. The colony morphology of selected isolates grown on the nutrient agar medium.

Isolates	Size	Shape	Color	Texture	Edge
MWS-5	Large	Circular	White	Smooth	Circular
MWS-4	Medium	Circular	White	Raised	Circular
MWS-1	Large	Circular	White	Raised	Circular
CWS-4	Large	Circular	Yellow	Raised	Circular
CWS-5	Small	Circular	Creamy	Smooth	Circular
PWS-2	Large	Circular	Creamy	Flat	Circular
PWS-4	Large	Circular	Yellow	Smooth	Circular
PWS-5	Medium	Circular	Creamy	Smooth	Circular
PWS-3	Medium	Circular	Creamy	Raised	Circular
MWS-3	Small	Circular	Creamy	Raised	Circular
PWS-1	Small	Circular	Creamy	Raised	circular
CWS-1	Medium	Circular	White	Flat	Wrinkled
CWS-2	Small	Circular	Yellow	Raised	Circular
CWS-3	Small	Circular	Yellow	Raised	Circular
MWS-2	Large	Circular	Creamy	Raised	Circular
MWS-2*	Large	Circular	White	Raised	Circular
PWS-5*	Small	Circular	Creamy	Flat	Circular

Legend: MWS; milk waste soil[1], PWS; poultry waste soil[2], CWS; cafeteria waste soil[3], numbers represent the soil sample where bacteria were isolated.

Of these, 17 (10.6%) isolates were protease-producing bacteria, and an equal number of six (35.3%) isolates were obtained from each dairy and poultry waste-dumped soils, followed by five (29.4%) isolates from cafeteria waste-dumped soil, indicating that proteolytic enzyme-producing bacteria were distributed across all types of collected soil. Among the 17 isolates, 11 (64.7%) were effective protease producers and showed the largest (>15 mm) zone of inhibition on skim milk agar medium (Table 2). However, Hyseni et al. (2020) reported 66 protease-positive bacterial isolates from different soil types, but only five (7.6%) isolates were effective in protease production. Similarly, six bacterial isolates from fish market waste soil were reported to be effective in protease production (Ravishankar, 2012).

On the other hand, Hadush et al. (2017) identified 85 effective protease-producing bacterial isolates from soils.

Footnote: [1] Topsoil (10–15 cm) collected from milk waste and washout dumping sites
[2] Topsoil (10–15 cm) collected from poultry waste and washout dumping sites
[3] Topsoil (10–15 cm) collected from cafeterial waste and washout dumping sites

Figure 1. The representative colonies of bacterial isolates recovered from soil samples

These protease-producing bacteria varied in colony size, color, and texture, although all of the isolates displayed a circular colony shape with defined edges (Table 1 and Figure 1). Most isolates had large colonies (41.2%), creamy color (47%), and a raised texture (58.5%), while a notable portion (29.4%) exhibited white colonies with a smooth texture and medium to small sizes. This differs from other reports (Bhowmik et al., 2015).

3.2. **Primary screening of protease-producing bacteria**

In this study, 17 isolates demonstrated protease production potential on skim milk agar medium and exhibited different zones of inhibition (Table 2). These isolates were significantly more numerous compared to six protease-producing bacteria screened from the gut of shrimp (Bhowmik et al., 2015). They were categorized into three groups based on when they produced the enzyme, depending on the bacterial incubation period. The first group includes three (27.3%) isolates that started producing protease within 24 hours of incubation and showed zones of inhibition ranging from 11.3 to 14.3 mm. The second group consists of five (45.5%) isolates that produced protease within 48 hours and showed zones of inhibition from 10.7 to 18 mm. The third group contains three (27.3%) isolates that produced protease after 72 hours of incubation, with zones of inhibition measuring 15 to 19 mm (Table 2). This indicates that the protease production potential varies among isolates or species, depending on bacterial growth timing, consistent with findings from other studies (Bhowmik et al., 2015; Hyseni et al., 2020). These results align with well-documented reports in the literature on protease-producing bacteria and enzyme production (Singh et al., 2015).

Table2. Diameter of the zone of inhibition in mm formed by isolates in skim milk agar medium within various incubation periods

Isolates	24hours	48hours	72hours
MWS-5	14.3 ± 0.94^{a}	19.3 ± 0.94^{b}	28 ± 1.63^{b}
MWS-4	11.3 ± 0.47^{b}	29.3 ± 2.49^{a}	39.7 ± 4.11^{a}
CWS-4	13.3 ± 2.62^{a}	14.3 ± 1.7^{c}	25 ± 2.16^{c}
MWS-1	*	13.3 ± 1.25^{c}	17 ± 0.82^{e}
CWS-5	*	13 ± 1.41^{c}	19.3 ± 0.94^{d}
PWS-2	*	18 ± 2.83^{b}	24.7 ± 3.77^{c}
PWS-4	*	17 ± 0.00^{b}	24.3 ± 7.59^{c}
PWS-5	*	10.7 ± 0.94^{d}	19 ± 1.41^{d}
PWS-3	*	**	15 ± 0.00^{f}
MWS-3	*	**	15 ± 3.56^{f}
PWS-1	*	***	19 ± 1.41^{d}

Legends: MWS; milk waste soil, PWS; poultry waste soil, CWS; cafeteria waste soil, numbers represent the sample number where bacteria were isolated, *; isolates showed highlights of enzyme production, **, isolates showed modestly visible enzyme production, ***, isolates showed clearly visible but un-measurable enzyme production, the superscript letter in the same column shows a significant difference of results

An early protease-producing isolate or first group of bacteria, MWS-5, MWS-4, and CWS-4, showed a steady increase in the zone of inhibition, such as 19.3 mm, 29.3 mm, and 14.3 mm at 48 hours, and 28 mm, 39.7 mm, and 25 mm at 72 hours of incubation, respectively (Table 2). Similarly, the two isolates (PWS-2 and PWS-4) from the second group of bacteria showed significant zones of inhibition, measuring 18 mm and 17 mm at 48 hours, and 24.7 mm and 24.3 mm at 72 hours, respectively. This result was consistent with a 19 mm zone of inhibition exhibited by the bacterial isolate (ML-12), screened from the effluent of Modjo leather industry within 24 hours of incubation (Masi et al., 2021). However, the third group of bacteria demonstrated a slower protease production ability, with zones of inhibition ranging from 15 to 19 mm after 72 hours of incubation(Table 2). Bacterial isolates with the smallest zones of inhibition (12 mm, 14 mm, and 18 mm), indicating lower protease production potential, have also been reported in other studies (Bhowmik et al., 2015; Masi et al., 2021).

More importantly, five isolates, such as MWS-4, MWS-5, CWS-4, PWS-2, and PWS-4, showed >20 mm proteolytic activities on the skim milk agar medium after 72 hours of incubation. These isolates were effective in protease production and exhibited zones of inhibition measuring 39.7 mm, 28 mm, 25 mm, 24.7 mm, and 24.3 mm, respectively (Table 2).

Notably, isolate MWS-4 demonstrated the highest rate (39.7 mm) of proteolytic activity on casein degradation, followed by isolates MWS-5 and CWS-4 with inhibition zones of 28 mm and 25 mm in diameter, respectively (Figure 2). Isolate MWS-4 proved more effective in proteolytic enzyme production and showed a 9.7 mm increase in casein degradation compared to isolate S1, which exhibited a 30 mm zone of inhibition (Bhowmik et al., 2015).

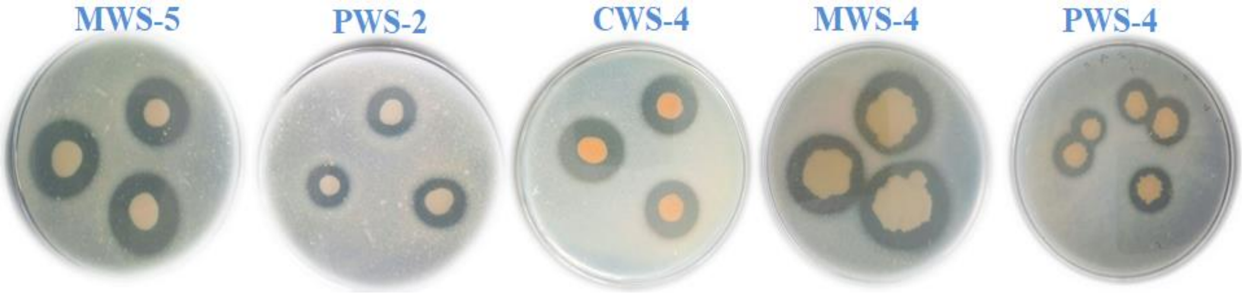

Figure2. Screened protease-producing bacterial isolates showed clear zone formation on skim milk agar medium

On the other hand, six (45.5%) isolates showed a 15–19 mm zone of inhibition after 72 hours of incubation and were considered less effective in protease production (Table 2). Therefore, only five potentially effective protease-producing isolates (MWS-5, MWS-4, CWS-4, PWS-2, and PWS-4) were subjected to re-screening based on the effect of crude protease extract and their cellular and physicochemical characteristics.

3.3.Rescreening of effective isolates

The proteolytic activities and enzyme production efficiency of the five isolates (MWS-5, MWS-4, PWS-2, PWS-4, and CWS-4) were further assessed using crude protease extracts (Figure 3). The isolates demonstrated a correlation between proteolytic activity and enzyme production performance (Table 3). Three isolates, MWS-5, PWS-4, and CWS-4, showed proteolytic activities of 15.4 mm, 14.2 mm, and 13.6 mm within 24 hours of incubation, producing 172 U/mL, 160 U/mL, and 140 U/mL of protease enzyme, with relative enzyme activities of 95.5%, 88.9%, and 77.8%, respectively (Table 3). The highest proteolytic activity (18.5 mm) was observed in PWS-2, which also produced a high level (180 U/mL) of protease enzyme. This represented the best proteolytic performance among the isolates, surpassing the results seen in the cultural experiment (Table 2). In contrast, MWS-4 exhibited lower proteolytic activity (11.8 mm) and enzyme production (110 U/mL), with a reduced relative activity of 61.1%, indicating lower effectiveness compared to the proteolytic activity observed during the cultural test (Table 2). Despite variations in casein degradation and enzyme output, all isolates demonstrated promising proteolytic activity and crude protease enzyme quantification using tyrosine standard (Figure 3).

Table 3. Diameter of the zone of inhibition in mm formed by the extracted protease enzyme on skim milk agar medium, and enzyme quantification using the tyrosine standard method.

Sources	Isolates	Inhibition zone of extracted protease	Protease activities	Relative activities (%)
Milk waste soil	MWS-5	15 mm	172 U/mL	95.5
Milk waste soil	MWS-4	11.8 mm	110 U/mL	61.1
Poultry waste soil	PWS-2	18.5 mm	180 U/mL	100
Poultry waste soil	PWS-4	14.2 mm	160 U/mL	88.9
Cafeteria waste soil	CWS-4	13.6 mm	140 U/mL	77.8

Legends:U, amount of enzyme that catalyses the reaction of 1 mmol of substrate per minute

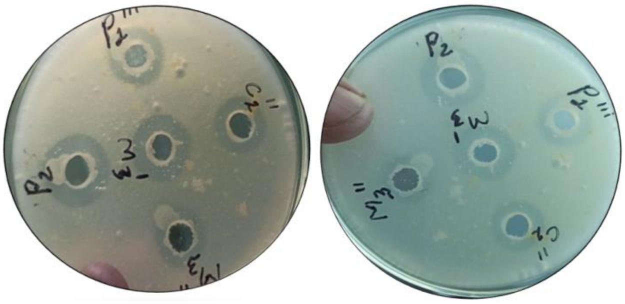

Figure3. Inhibition zone of extracted protease enzyme from screened bacterial isolates on the skim milk agar medium. NB: P1''' = PWS-4, P2= PWS-2, M3' = MWS-4, M3''= MWS-5

3.4. Gram reaction and cellular characteristics of the isolates

Based on bacterial cell wall composition, all the screened isolates (MWS-5, MWS-4, CWS-4, PWS-2, and PWS-4) retained the purple color of the primary stain (crystal violet) and confirmed they are gram-positive bacteria (Figure 4). Although they were gram-positive, variation in cell shape, cell arrangement, and the amount of biomass produced was observed. For instance, the two isolates (PWS-2 and PWS-4) obtained from poultry waste soils were rod-shaped single bacteria, but the milk waste soil isolate, MWS-5, and the cafeteria waste soil isolate, CWS-4, were coccoid bacteria with a single cell arrangement (Table 4). Only one isolate (MWS-4) from milk waste soil was a diplococcus in cell arrangement. This shows that protease-producing bacteria can be categorized into different species based on cellular and structural characteristics. The Gram-negative and Gram-positive protease-producing bacteria with rod and coccoid shapes and chained or clustered cell arrangements have been reported from the gut of shrimp (Bhowmik et al., 2015).

Furthermore, these isolates also showed significant ($P < 0.05$) variation in biomass production after 24 hours of incubation (Table 4). The isolate CWS-4 grew considerably faster and produced a high amount of biomass (60.95 mg), followed by PWS-4, which produced a substantial (39.4 mg) amount of biomass, outperforming the other isolates that had intermediate biomass production levels (32.5 and 23.9 mg). The lowest biomass (21.4 mg) was recorded by PWS-2 compared to the others. In a similar study, effective protease-producing bacteria with varying levels of biomass production were reported (Masi et al., 2021).

Table4. The Gram reaction, cell shape, cell arrangement, and biomass production of selected protease-producing bacterial isolates

Isolates	Shape	Arrangement	Gram reaction	Biomass in mg
PWS-2	Rod	Single	Positive	21.4 ± 3.3^{e}
PWS-4	Rod	Single	Positive	39.4 ± 0.85^{b}
MWS-4	Diplocci	Pair	Positive	23.9 ± 4.9^{d}
MWS-5	Cocci	Single	positive	32.5 ± 8.5^{c}
CWS-4	Cocci	Single	Positive	60.95 ± 4.64^{a}

Legend: The superscript letter in the same column shows an insignificant difference in results

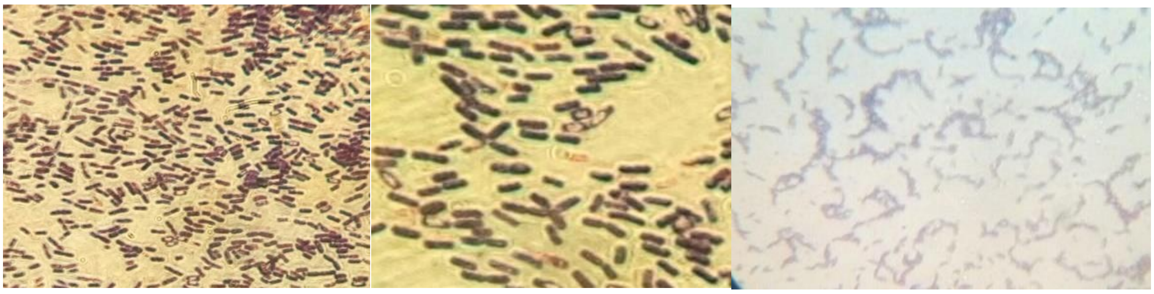

Figure 4. The Gram reaction results of selected protease-producing bacterial isolates.

3.5.Physicochemical characteristics of protease-producing bacteria

3.5.1. Temperature tolerance

The temperature tolerance of all five isolates is shown in Table 5. Most isolates grew well at temperatures from 5°C to 35°C, except CWS-4, which did not grow at 35°C within 24 hours of incubation. Three isolates—MWS-4, MWS-5, and PWS-4—were effective in growth across the temperature range of 5°C to 35°C, indicated by the +++ designation, suggesting they may grow well at ambient temperatures. In contrast, CWS-4 showed intermediate (++) growth at 5°C but high (+++) growth at 30°C, without growing at temperatures above 30°C.

Table 5. The temperature tolerance potential of protease-producing bacterial isolates

Isolates	Temperature ranges				
	5 ^{0}C	30 ^{0}C	35 ^{0}C	40 ^{0}C	45 ^{0}C
MWS-4	+++	+++	+++	--	--
MWS-5	+++	+++	+++	--	--
CWS-4	++	+++	--	--	--
PWS-4	+++	+++	+++	--	--
PWS-2	++	++	++	+++	--

Legends: +++, represents high growth of bacteria; ++, intermediate growth of bacteria; --, non-growth of bacteria

However, the isolate PWS-2 was different in temperature tolerance and showed moderate (++) growth at temperatures between 5 °C and 35 °C, and high (+++) growth at 40°C. This isolate might be temperature-tolerant (Table 4). Generally, 80 % of isolates failed to grow at temperatures above 40°C. This suggests that the optimal incubation temperature for protease-producing bacteria in this study is likely not to exceed 35 °C, except for isolate PWS-2, which can grow up to 40°C. Reports also demonstrated the growth of protease-producing bacteria over a different range of temperatures (Bhowmik et al., 2015).

3.5.2. Salt tolerance

The growth of protease-producing bacteria at different concentrations of NaCl showed variation over 24 hours of incubation (Table 6). All isolates grew at salt concentrations ranging from 0.5% to 6%, but at different rates. Notably, three isolates, PWS-4, MWS-4, and MWS-5, exhibited consistent growth across all tested NaCl concentrations, producing a high (+++) number of colonies (Table 6). In contrast, isolate PWS-2 showed slow growth with fewer (+) colonies at NaCl levels between 0.5% and 1.5%, but it grew rapidly (+++) at 3% to 5.5% NaCl, then slowed (++) at 6%. This suggests that PWS-2 might be slightly halo-philic and can grow in salt concentrations below 6% (Amoozegar, et al., 2003).

Table 6. The salt tolerance potential of protease-producing bacterial isolates

Isolates	Salt concentration (%)											
	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6
PWS-2	+	+	+	++	++	+++	+++	+++	+++	+++	+++	++
PWS-4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
MWS-4	+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++	+++
MWS-5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

CWS-4	++	++	++	++	++	++	+	--	--	--	--	--

Legends: +++, represents high growth of bacteria; ++, intermediate growth of bacteria; +, low growth of bacteria; --, non-growth of bacteria

However, isolate CWS-4 was grown at NaCl concentrations ranging from 0.5% to 3.5%, but it never grew at salt concentrations above 3.5%. Researchers classified protease-producing bacteria into three groups: slight halo-philes, which can grow at 2% to 5% NaCl; moderate halo-philes, which can grow at 5% to 20% NaCl; and extreme halo-philes, which can grow at 20% to 30% NaCl, considered the optimal salt concentration (Amoozegar et al., 2003). On the other hand, the effect of salt concentrations in the range of 0% to 2% was not significant on bacterial growth and protease production, with high enzyme activity observed at 1% NaCl concentration (Suganthi et al., 2013).

3.5.3. PH tolerance

The pH level of the medium mainly affects bacterial growth. In this study, the protease-producing bacteria inoculated onto nutrient agar media adjusted to different pH levels showed variation in their growth rates (Table 7). The two bacterial isolates from milk waste soil (MWS-4 and MWS-5) grew across a broad pH range from 4 to 9. Their growth improved at pH 5.5 to 9(Table 7). Suleiman et al. (2020) reported an optimal pH of 7 to 8 for protease-producing bacteria growth. Similarly, the poultry waste soil isolate, PWS-4, showed high growth at pH 5.5 to 9, moderate growth at pH 4.5 to 5, and did not grow at pH 4. Conversely, PWS-2 did not grow in acidic or basic conditions but only under neutral conditions. This indicates that PWS-2 is neither alkalo-philic nor acidophilic but might be a neutro-philic bacterium. Recent studies also report the presence of neutro-philic protease-producing bacteria (Gómez-Alonso et al., 2022).

Table 7. The pH tolerance of protease-producing bacterial isolates grown for 24 hours of incubation

Isolates	pH values used for the test					
	4	4.5	5	5.5	8	9
PWS-2	--	--	+	++	+	--
PWS-4	--	++	++	+++	+++	+++
MWS-4	++	++	++	+++	+++	+++
MWS-5	++	++	++	+++	+++	+++
CWS-4	--	+	+	++	+++	_ _

Legends: +++, represents high growth of bacteria; ++, intermediate growth of bacteria; +, low growth of bacteria; --, non-growth of bacteria

On the other hand, cafeteria waste soil isolate CWS-4 was less (+ or -) tolerant to acidic conditions but grew better at neutral conditions and was abundantly (++) grown at a pH of 8, failing to grow at pH 9. This suggests it might be a slightly alkalo-philic bacterium (Table 7). Different researchers have recorded the optimal growth of proteolytic enzyme-producing bacteria at various pH values, such as pH 7.8 (Tsujibo et al., 1990), 8.0 (Anwar and Saleemuddin, 1997), and 8.5 (Anbu et al., 2013).

4. Conclusion

This study focused on isolating and screening protease-producing bacteria from soils contaminated with dairy, poultry, and cafeteria waste. Of the 160 bacterial isolates, 17 were protease-positive. Among these, eleven isolates were identified as effective protease producers and showed significant proteolysis around their colonies on skim milk agar plates. Further screening revealed that three isolates (PWS-2, PWS-4, and MWS-5) produced the highest (180 U/mL, 172 U/mL, and 160 U/mL, respectively) protease in relative to others. Morphological and cellular characterization indicated that isolates PWS-2 and PWS-4 were rod-shaped, gram-positive bacteria, and isolate MWS-5 was a coccoid bacterium. These isolates were further grown at a range of temperatures, pH, and salt concentrations, producing substantial biomass (21.4–39.4 mg). However, further molecular-level identification, optimization of growth and production conditions, and characterization of the resulting protease will need further investigation.

Author contribution statement

Birhan Aynalem: conceptualization, design, data analysis, supervised, reviewed, and edited the paper. EmebeteHirpesa and Rehima Muhdin: conceptualization, conducted lab experiment, wrote original draft. Aklilu Menber and Birhanu Tadele: helped with lab workand edited the manuscript.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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Declaration of conflict of interest

The authors declared that they have no conflict of interest regarding this paper.

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