ORIGINAL ARTICLE

Optimization of Fermentation Condition for Hydrolytic Enzyme Production by Endophytic Bacteria from *Kleinhovia hospita* L.

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ABSTRACT

Kleinhovia hospita L. is a tropical Indonesian medicinal plant that hosts endophytic bacteria capable of producing hydrolytic enzymes. However, studies exploring the enzymatic potential of endophytic bacteria associated with *K. hospita* L. remain limited. This study evaluated hydrolytic enzyme produced by endophytic bacteria isolated from *K. hospita* L. under different fermentation pH (5, 7, and 10) and temperatures (28, 34, and 40 °C). Enzyme activity was determined using the well diffusion method, and protein content was measured using the biuret method with BSA as a standard. The highest protein concentration was recorded at pH 10 in isolate KHB1 (607.5 µg/mL) and at 40 °C in isolate KHD3 (1052.5 µg/mL). Enzyme activities varied among isolates, pH and fermentation conditions. The highest amylase activity was observed in isolate KHD2 (12.80 ± 0.24 mm). Pectinase and esterase activities were highest in isolates KHD1 (20.02 ± 4.08 mm) and KHD3 (18.11 ± 0.82 mm), respectively. The largest lipase activity was detected in isolate KHA1 (39.48 ± 3.74 mm), whereas the highest cellulase and protease activities were observed in isolate KHD1. These results indicate that fermentation pH and temperature influence hydrolytic enzyme production by endophytic bacteria from *K. hospita* L.

Keywords: *Kleinhovia hospita* L.; Hydrolytic enzyme; Endophytic bacteria; pH fermentation; Temperature fermentation

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Introduction

Kleinhovia hospita L. is a tropical medicinal plant widely distributed in Indonesia and traditionally used as a vegetable and herbal remedy for various health benefits [1]. Like many medicinal plants, *K. hospita* L. hosts diverse endophytic bacteria that inhabit internal plant tissues without causing apparent harm to their host [2]. These microorganisms have attracted increasing attention because they are known to produce various bioactive compounds and enzymes that may contribute to plant metabolism and offer potential applications in biotechnology [3,4]. Among microbial products, hydrolytic enzymes such as amylase, cellulase, protease, lipase, pectinase, and esterase play important roles in catalyzing the degradation of complex organic compounds into simpler molecules. These enzymes are widely used in various biotechnological processes, including food processing, pharmaceuticals, and bioethanol production. Endophytic microorganisms isolated from medicinal plants have been reported as promising sources of hydrolytic enzymes due to their ability to adapt to diverse environmental conditions and produce metabolically active compounds [5,6]. Several studies have reported hydrolytic enzyme production from endophytic bacteria isolated from different plant species. However, research on endophytic bacteria associated with *K. hospita* L. remains very limited. Most previous studies on *K. hospita* L. have focused primarily on its phytochemical composition and pharmacological properties, such as antioxidant, anti-

inflammatory, and antimicrobial activities [7,8]. Information regarding the enzymatic potential of endophytic bacteria inhabiting this plant, particularly their ability to produce hydrolytic enzymes, is still scarce. Enzyme activity can be evaluated using pure enzyme extracts or bacterial isolates to determine their catalytic potential [9].

The production and activity of enzyme proteins are strongly influenced by fermentation condition [10,11]. Therefore, understanding the effects of these culture conditions is crucial for optimizing enzyme production and application. The influence of fermentation conditions such as pH and temperature on hydrolytic enzyme production by endophytic bacteria from *K. hospita* L. has not been comprehensively investigated. Since *K. hospita* L. grows in tropical environments, its associated endophytic bacteria may possess unique metabolic capabilities and adaptability to environmental fluctuations, making them promising candidates for enzyme production.

Therefore, this study aims to isolate and characterize endophytic bacteria from different tissues of *K. hospita* L. and evaluate their potential to produce hydrolytic enzymes, including amylase, cellulase, protease, lipase, pectinase, and esterase. In addition, this study investigates the effects of fermentation pH and temperature on enzyme activity. This work provides new insights into the enzymatic potential of endophytic bacteria associated with *K. hospita* L. and contributes to the exploration of microbial resources for hydrolytic enzyme production.

METHODS

Materials

The materials used in this study were *K. hospita* L. plants were collected from Uwemanje Village, Kinovaro District, Sigi Regency, Central Sulawesi, Indonesia. The plant was taxonomically identified at the Plant Biosystematics Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Tadulako University, Indonesia (ID: 410/UN28.1.28/BIO/2023). Nutrient Agar and Nutrient Broth (Merck), distilled water, 70% ethanol. Protein analysis employed bovine serum albumin (BSA; Thermo Scientific) and biuret reagent (ROFA). Sodium hypochlorite and iodine solution (Merck) were used for surface sterilization.

The culture media and enzyme assay reagents included yeast extract, peptone, agar, NaCl, ammonium sulfate, $NaNO_3$, KCl, $CaCl_2{\cdot}2H_2O$, Na_2HPO_4, KH_2PO_4, K_2HPO_4 (Merck), pectin (Sigma), skim milk powder, Tween 20 (Merck), Congo red, and cetyltrimethylammonium bromide (CTAB; Sigma).

Sample Preparation

Leaves, steam, and roots of *K. hospita* L. were washed under running water, cut into segments of approximately 1 cm, and surface-sterilized sequentially using 70% ethanol for 1 min, 5.25% NaClO for 3 min, and 70% ethanol for 30 s, followed by three rinses with sterile distilled water. The samples were then placed on Nutrient Agar (NA) and incubated at 37 °C for 24 h until endophytic bacterial colonies appeared [12].

Identification of Endophytic Bacteria

Endophytic bacteria were identified microscopically through Gram staining. Bacterial isolates were heat-fixed on glass slides using physiological saline, stained sequentially with crystal violet and iodine for 1 min each, decolorized with 90% ethanol for 30 s, counterstained with safranin for 1 min, and observed under a light microscope at 1000× magnification [13].

Fermentation of Endophytic Bacteria

Endophytic bacterial fermentation was performed using liquid fermentation in Nutrient Broth (NB) medium with variations in temperature and pH. One loopful of pure endophytic bacterial isolate from the stock culture was inoculated into 200 mL of sterile NB medium in Erlenmeyer flasks. For temperature variation, the cultures were incubated in a shaker incubator at 150 rpm for 48 h at 28 °C, 34 °C, and 40 °C, while the pH of the medium was maintained at pH 7. For pH variation, the NB medium was adjusted to pH 5, 7, and 10 using a pH meter prior to sterilization. The cultures were then incubated at a constant temperature of 34 °C for 48 h at 150 rpm to evaluate the optimization of pH on enzyme production. After fermentation, the cultures were centrifuged at 12,000 rpm for 15 min at 4 °C to separate the supernatant and cell debris. The

resulting supernatant was collected and used for protein content determination and subsequent assays of hydrolytic enzyme [11,14].

Protein Content Determination

Protein content was determined using the biuret method with BSA as the standard. A BSA stock solution (10,000 µg/mL) was prepared and diluted to obtain concentrations of 50–1,000 µg/mL for standard curve construction. Each standard and sample (1 mL of endophytic bacterial supernatant) was mixed with 2 mL of biuret reagent and incubated for 30 min at room temperature. Absorbance was measured at 513 nm using a UV–Vis spectrophotometer, and protein content was calculated based on the BSA standard curve. All measurements were performed in triplicate, and the results were expressed as mean ± standard deviation [15,16].

Hydrolytic Enzyme Activity Assay

Amylase activity was assayed on agar medium containing 1% (w/v) starch. After sterilization at 121 °C for 15 min, endophytic bacterial supernatant was added to wells and incubated at 25 °C for 48 h. Iodine solution was then applied to the agar surface, and the formation of a clear zone indicated amylase activity [17,18].

Pectinase activity was evaluated using agar medium containing yeast extract (2 g), ammonium sulfate (2 g), Na_2HPO_4 (6 g), KH_2PO_4 (3 g), pectin (5 g), and agar (15 g) per liter. The medium was sterilized at 121 °C for 15 min, inoculated with bacterial supernatant, and incubated at 25 °C for 24 h. CTAB solution was added to the agar surface, and clear zone formation indicated pectinase activity [19,20].

Esterase activity was determined on agar medium containing peptone (10 g/L), NaCl (5 g/L), $CaCl_2{\cdot}2H_2O$ (0.1 g/L), agar (18 g/L), and Tween 80 (1% v/v, added after sterilization), adjusted to pH 7.0. Bacterial supernatant was added to wells and incubated at 28 °C for 120 h. Esterase activity was indicated by the formation of a white opaque zone around the wells [21,22].

Lipase activity was assayed on agar medium containing Nutrient Broth (8 g/L), $CaCl_2{\cdot}H_2O$ (0.1 g/L), agar (15 g/L), and Tween 20 (20 mL/L, added after sterilization), adjusted to pH 6.0. Plates were incubated at 25 °C for 48 h, and lipase activity was indicated by an opaque zone around the wells [21,22].

Cellulase activity was evaluated using CMC agar medium containing $NaNO_3$ (1 g/L), K_2HPO_4 (1 g/L), KCl (1 g/L), $MgSO_4$ (0.5 g/L), yeast extract (0.5 g/L), glucose (1 g/L), CMC (5 g/L), and agar (15 g/L). After incubation at 25 °C for 120 h, plates were flooded with 0.2% (w/v) Congo red for 20 min and washed with 5 M NaCl. A clear yellow zone against a red background indicated cellulase activity [23].

Protease activity was determined using nutrient agar supplemented with 1% (w/v) skim milk. After inoculation with bacterial supernatant, plates were incubated at 25 °C for 24 h. The presence of a clear zone around the wells indicated protease activity [24].

Enzyme activity was evaluated based on the diameter of the clear (or opaque) zone formed around the wells on the agar medium. The diameter of the hydrolysis zone was measured in millimeters using a digital caliper. All measurements were performed in triplicate, and the results were expressed as mean ± standard deviation.

Data Analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 10.6.1. Differences were considered statistically significant at $p < 0.05$.

RESULT AND DISCUSSION

A total of seven endophytic bacterial isolates were obtained from *K. hospita* L., consisting of three isolates from the roots (KHA), three from the leaves (KHD), and one from the stem (KHB), as shown in **Figure 1**. The higher number of isolates recovered from roots and leaves may be associated with the favorable microenvironment of these tissues for colonization. Roots are in direct contact with soil microbial communities and nutrient sources, which increases the likelihood of microbial entry and persistence within plant tissue as endophytes. In addition, roots release various organic compound such as sugars, amino acids,

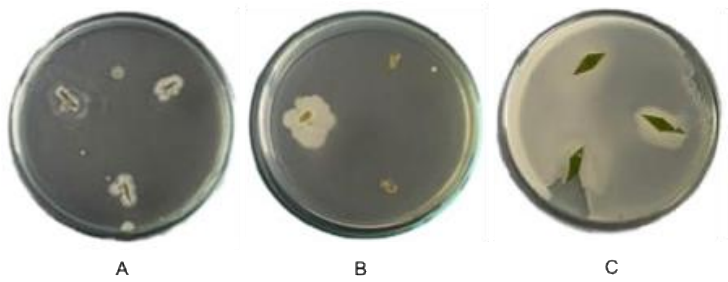

Figure 1. Endophytic bacteria isolated from *Kleinhovia hospita* L. A. Root, B. Stem, and C. Leaf

and organics acids that can promote microbial colonization and growth [25]. Leaves may also support endophytic bacterial communities due to the availability of nutrients, diverse ecological niches influenced by photosynthetic activity and plant metabolism, and suitable anatomical structures that facilitate microbial persistence [26]. All isolates were rejuvenated and cultured through liquid fermentation in NB to induce extracellular metabolite production. The fermentation broth was centrifuged to obtain the supernatant, which was subsequently used for enzyme activity assays. This approach is supported by previous studies reporting that fermentation supernatants of endophytic bacteria contain extracellular proteins, including hydrolytic enzymes [27]. Liquid fermentation enables bacterial growth and enzymes secretion into the surrounding medium, thereby facilitating the detection of extracellular enzymatic activity [28].

Macroscopic characteristics were initially observed to provide preliminary information on the morphology

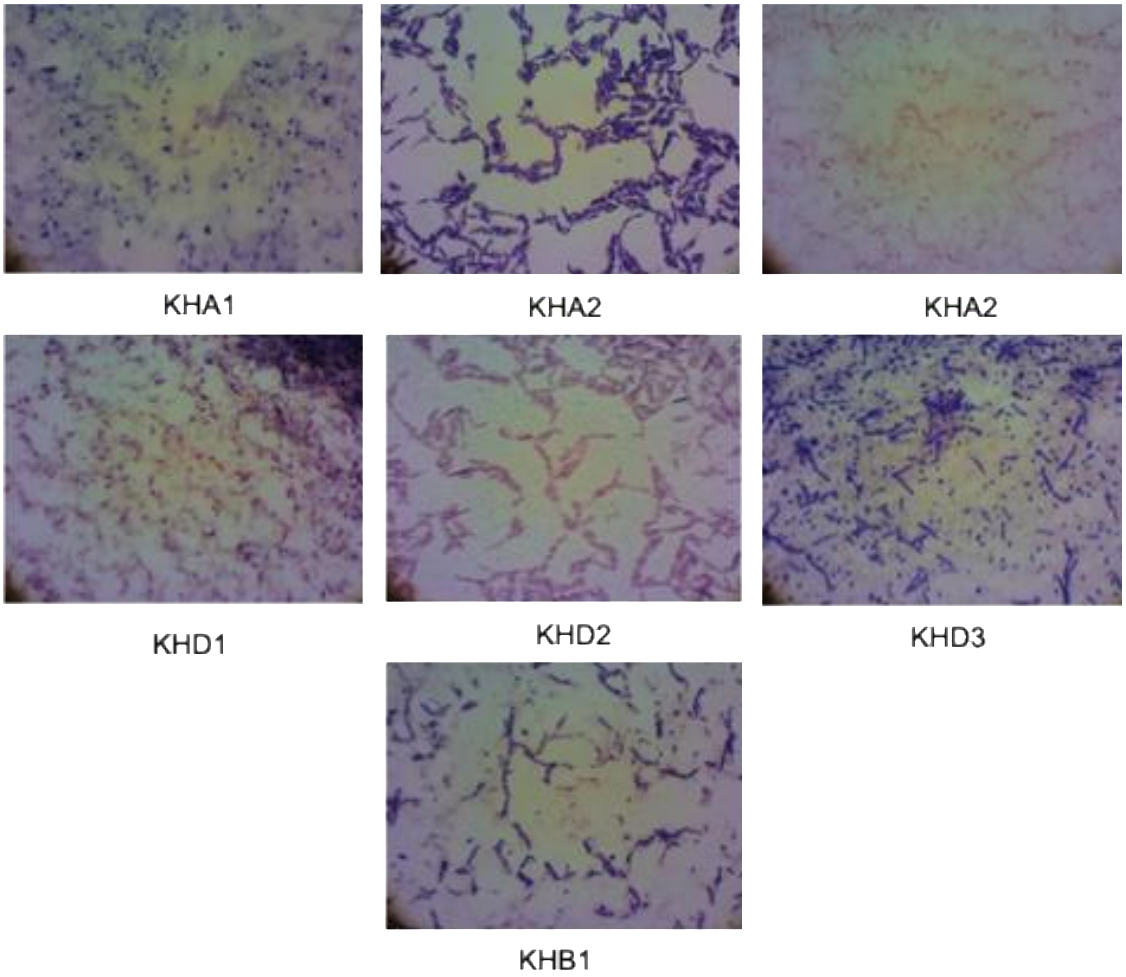

Figure 2. Microscopic characteristic of endophytic bacterial isolates *K. hospita* L. observed at 1000x magnification. (A-C) Root isolates, (D-F) Leaf isolates, and (G) Stem isolates

Table 1. Morphological characteristic of endophytic bacteria colonies

Isolate code	Colony shape	Margin shape	Elevation	Colony color	Colony Size
KHA1	Irregular	Undulate	Raised	White	Medium
KHA2	Irregular	Undulate	Raised	White	Medium
KHA3	Irregular	Undulate	Raised	White	Medium
KHB1	Irregular	Undulate	Raised	White	Medium
KHD1	Irregular	Entire	Raised	Yellow	Large
KHD2	Irregular	Entire	Raised	Yellow	Large
KHD3	Irregular	Entire	Raised	Yellow	Large

of bacterial colonies, including colony shape. Size, color, margin, elevation, and surface texture. The results of macroscopic observation are presented in **Table 1**. Microscopic identification using Gram staining showed that isolates KHA1, KHA2, KHB1, and KHD3 were Gram-positive, whereas KHA3, KHD1, and KHD2 were Gram-negative (**Table 2** and **Figure 2**). The presence of both Gram-positive and Gram-negative bacteria among the isolates reflects the diversity of endophytic bacterial communities associated with medicinal plants. Similar findings have been reported in other medicinal plants, where both Gram-positive and Gram-negative endophytic bacteria coexist within plant tissues. For example, a study on Stachys striata reported that 56% of endophytic isolates were Gram-positive and 44% were Gram-negative [29]. In general, Gram-positive endophytes such as Bacillus and Streptomyces are commonly dominant due to their ability to produce diverse bioactive metabolites and enzymes, while Gram-negative bacteria contribute to metabolic diversity and plant–microbe interactions [30]. These findings indicate that the coexistence of both bacterial groups is a common characteristic of endophytic communities in medicinal plants and supports the diversity observed in this study.

Table 2. Gram staining and cell morphology of endophytic bacterial isolates

Isolate code	Gram Staining	Cell shape
KHA1	+	Monococcus
KHA2	+	Streptococcus
KHA3	-	Monobacillus
KHB1	+	Streptobacillus
KHD1	-	Streptococcus
KHD2	-	Streptobacillus
KHD3	+	Streptococcus

Extracellular protein content analysis was performed using the biuret method with BSA as the standard. The biuret method measures protein concentration based on the formation of a colored complex between biuret reagent and peptide bonds [31,32]. Protein production by the endophytic bacterial isolates varied under different fermentation temperature and pH conditions, as shown in **Figure 3**.

Based on fermentation temperature, the highest protein concentrations at 28 °C, 34 °C, and 40 °C were produced by isolates KHA1 (962.5 µg/mL), KHD2 (560 µg/mL), and KHD3 (1052.5 µg/mL), respectively. Meanwhile, based on fermentation pH, the highest

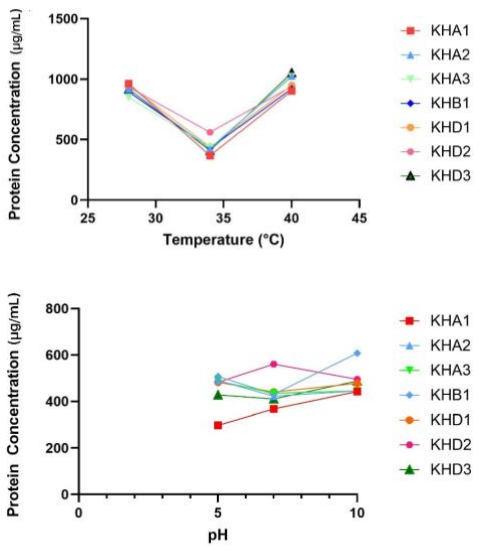

Figure 3. Protein concentration of endophytic bacteria *K. hospita* L. under temperature and pH variation. Stem, and C. Leaf

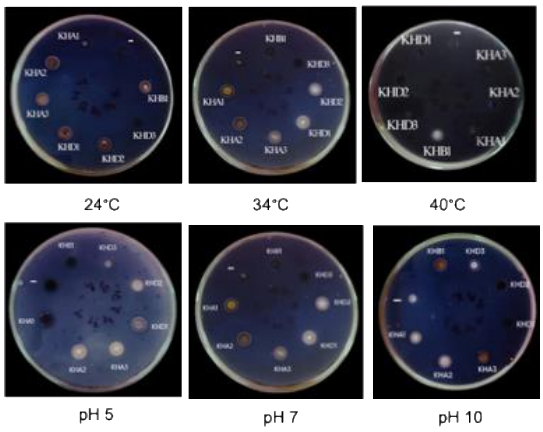

Figure 4. Endophytic bacteria isolated from *Kleinhovia hospita* L. A. Root, B. Stem, and C. Leaf

protein concentration at pH 5 was recorded in isolate KHD1 (507.5 µg/mL), whereas at pH 7 and pH 10, the highest protein contents were observed in isolates KHD2 (560 µg/mL) and KHB1 (607.5 µg/mL), respectively. These results indicate that fermentation temperature and pH play important roles in bacterial growth and extracellular protein production. Increasing temperature can enhance metabolic activity and enzyme secretion; however, excessively high temperatures may inhibit bacterial growth and cause protein denaturation, leading to reduced protein stability [33]. Similarly, appropriate pH conditions support optimal bacterial growth and metabolic activity, thereby promoting the secretion of extracellular proteins, including enzymes that contribute to the dissolved protein content in the fermentation supernatant [34]. In addition, variations in protein levels among isolates may be associated with differences in growth rate and metabolic efficiency during fermentation, which influence each isolate's capacity to synthesize and secrete extracellular proteins [35].

Amylase activity was evaluated using starch agar medium, as shown in **Figure 4**. The results indicated that the highest amylase activity was obtained in isolate KHD2 fermented at 34 °C, with a clear zone diameter of 12.80 ± 0.24 mm. At a fermentation temperature of 28 °C, isolates KHA1 and KHD3 did not exhibit amylase activity, whereas at 40 °C, detectable activity was observed only in isolate KHB1 (**Figure 5**). These results indicate that suggest that 34 °C provides more favorable condition for amylase production, likely because bacterial metabolic activity and enzyme synthesis occur more efficiently at this temperature. In contrast, lower temperatures may slow microbial metabolism and enzyme production, while higher temperatures reduce enzyme stability or cause partial enzyme denaturation [36]. Variations in amylase activity were also observed under different pH conditions. At pH 5, the highest average clear zone diameter (12.24 ± 1.02 mm) was produced by isolate KHD1. At pH 7, the largest clear zone diameter (12.79 ± 0.24 mm) was observed in isolate KHD2, whereas at pH 10, the maximum clear zone diameter (12.34 ± 0.28 mm) was produced by isolate KHA2 (**Figure 5**).

One-way ANOVA analysis indicated that amylase activity among the isolates did not differ significantly under different pH and temperature conditions ($p > 0.05$). Nevertheless, temperature variation appeared to influence enzyme expression in several isolates, as some isolates showed undetectable activity at certain temperatures, while under different pH conditions only a limited number of isolates exhibited negative activity. Amylase activity was detected under both acidic and alkaline conditions, the highest activity occurred at pH 7, indicating that neutral pH is the most optimal condition for amylase production by the tested

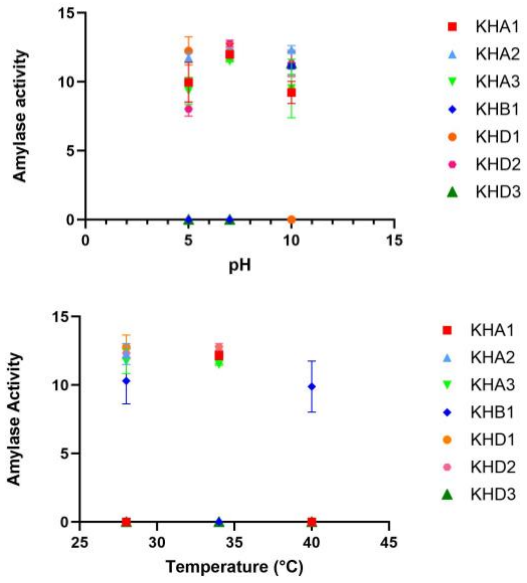

Figure 5. Amylase activity of endophytic bacteria of *K. hospita* L. under pH and temperature variation

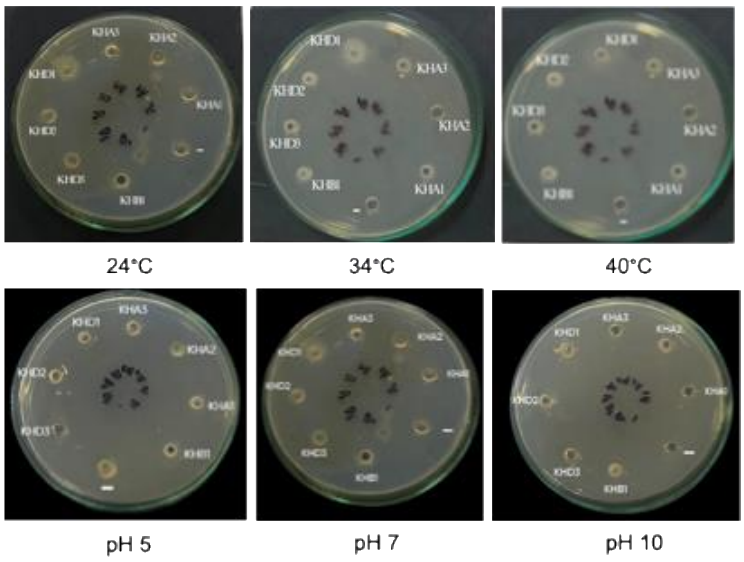

Figure 6 . Clear zone diameter of pectinase activity produced by endophytic bacteria isolated from *K. hospita* L. under temperature and pH variation.

endophytic bacteria [37]. These results demonstrate that fermentation temperature and pH play important roles in determining amylase activity in endophytic bacteria. Moderate temperatures support metabolic activity and enzyme stability, while neutral pH promotes optimal enzyme–substrate interactions [38]. This pattern is consistent with previous findings on Bacillus licheniformis, which reported maximum amylase activity at temperatures around 35 °C [39]. Differences in amylase activity among isolates reflect variations in metabolic capacity, enzyme expression levels, and substrate utilization [40]. Several amylase-producing isolates were identified as members of the genus Bacillus, which is well known for its high amylolytic potential [41,42]. Pectinase activity was indicated by the formation of clear zone around the colony after the addition of 1% CTBA, as illustrated in **Figure 6**. Based on pH and temperature variation assays, as presented in **Figure 7**, pectinase activity was detected exclusively at pH 7, with an average clear zone diameter of 14.40 ± 7.21 mm produced by isolate KHD1, while activity was not observed at other pH levels. The absence of clear zones under acidic and alkaline conditions indicates that neutral pH provided the most favorable environment for pectinase production by the endophytic bacterial isolates examined in this study. This observation is consistent with previous reports showing that pectinase exhibits optimal activity across a broad pH range (3.5–11), with maximum activity commonly occurring near neutral pH [43]. The lack of detectable activity at other pH values may be attributed to reduced extracellular pectinase secretion under unfavorable pH conditions, resulting in insufficient hydrolysis of the pectin substrate [44]. Fermentation temperature also had a significant effect on pectinase activity. Detectable activity was observed only in isolate KHD1, with the highest activity recorded at 34 °C, as indicated by a clear zone diameter of 20.02 ± 4.08 mm. Under pH and temperature variation conditions, pectinase activity was detected only in one isolate, suggesting that this enzymatic capability is limited to specific endophytic bacteria. Consequently, statistical comparison among isolates could not be performed, and the results were interpreted descriptively.

This result suggests that moderate fermentation temperatures favor pectinase production, whereas lower or higher temperatures may limit enzyme synthesis or reduce enzyme stability. Similar temperature-dependent trends have been reported previously by Shrestha et al., in which pectinase activity increased with temperature

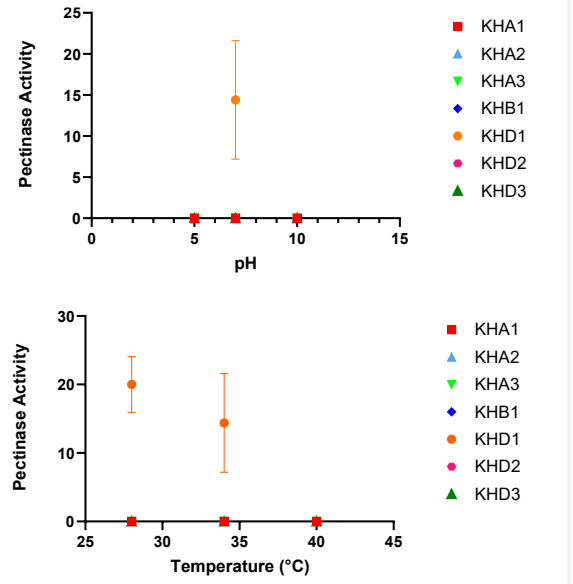

Figure 7. Pectinase activity of endophytic bacteria of *K. hospita* L. under pH and temperature variation.

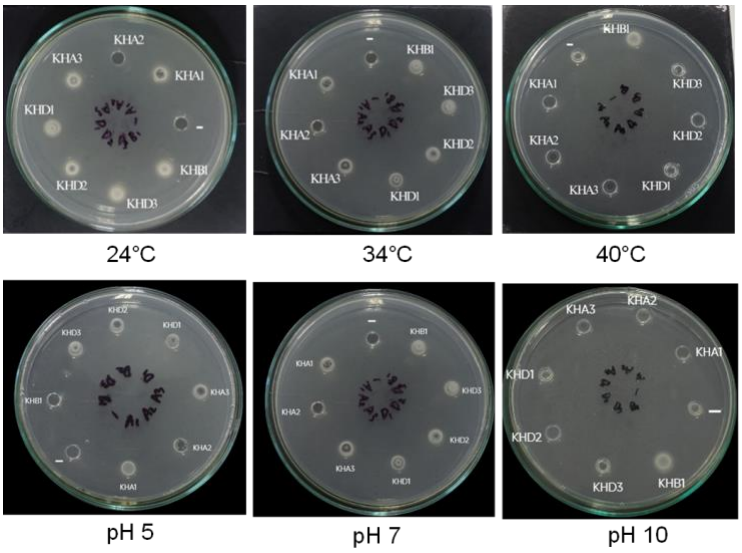

Figure 8. Clear zone diameter of esterase activity produced by endophytic bacteria isolated from *K. hospita* L. under temperature and pH variation.

up to an optimum of approximately 35 °C and declined at higher temperatures due to enzyme denaturation or reduced catalytic efficiency [45]. The limited pectinase activity observed in the remaining isolates may be influenced by several factors, including restricted substrate availability, nutrient depletion, and isolate-specific metabolic differences. As bacterial populations increase, competition for available substrates can reduce enzyme production, particularly when carbon sources become limiting [19]. In addition, unfavorable environmental conditions may alter enzyme conformation, thereby impairing substrate binding at the active site and ultimately reducing catalytic performance [46].

Following the detection of esterase activity in the fermentation supernatants, the effects of pH and temperature variations on enzyme production were further evaluated in **Figure 8**. **Figure 9** shows that, based on pH variation, esterase activity was detected exclusively at pH 10, with detectable activity observed only in isolate KHB1, whereas activity was not observed at lower pH values. Therefore, statistical analysis could not be performed, and the results were interpreted descriptively. These results indicate that alkaline conditions favored esterase production by the endophytic bacterial isolates examined in this study. This finding is consistent with previous reports showing that the optimal pH range for esterase activity lies between pH 7.0 and 10.0 [47]. In addition to pH, esterase activity may also be influenced by salt concentration in the growth medium, as NaCl has been reported to enhance esterase activity at concentrations ranging from 5% to 25% [48]. Fermentation temperature also had a pronounced effect on esterase activity, as shown in **Figure 9**. Detectable activity was observed only at a fermentation temperature of 28 °C, at which all isolates except KHA2 exhibited esterase activity. The highest activity was recorded in isolate KHD3, with a clear diameter of 18.11 ± 0.82 mm. Under fermentation temperature variation, esterase activity was detected only at a single temperature condition. Nevertheless, among the isolates exhibiting positive activity, differences were not statistically significant based on one-way ANOVA ($p > 0.05$). Esterase activity was not detected at higher fermentation temperatures, suggesting possible inhibition of enzyme synthesis or reduce enzyme stability. Similar temperature-dependent patterns have been reported previously, with maximum esterase activity commonly

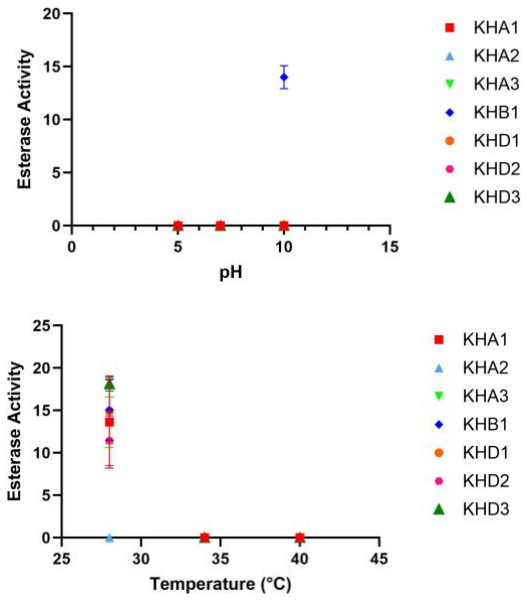

Figure 9. Esterase activity of endophytic bacteria of *K. hospita* L. under pH and temperature variation.

observed at fermentation temperatures around 30 °C [49]. Variations in esterase activity among isolates may also be attributed to intrinsic genetic differences. Differences in genome sequences among endophytic bacterial isolates can influence metabolic pathways, enzyme expression levels, and secretion efficiency, resulting in distinct enzymatic profiles even among isolates originating from the same host plant [50].

A clear zone formed on agar medium indicates positive lipolytic activity, resulting from the hydrolysis of lipids (triglycerides) into soluble fatty acids and glycerol, as illustrated in **Figure 10**. Lipase activity varied among the endophytic bacterial isolates under different pH and fermentation temperature conditions, as presented in **Figure 11**. Based on pH variation assays, lipase activity was detected only in isolate KHA1 at pH 5 (39.48 ± 3.74 mm) a nd in isolate KHA3 at pH 7 (27.44 ± 7.91 mm), whereas at pH 10, lipase activity was observed in six isolates, with an average opaque zone diameter of 17.77 ± 0.49 mm.

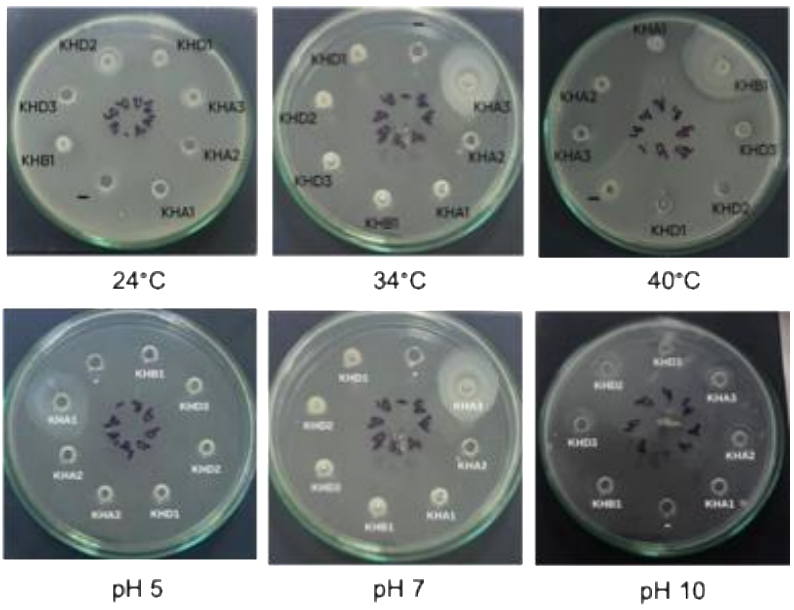

Figure 10. clear zone diameter of lipase activity produced by endophytic bacteria isolated from K. hospita L. under temperature and pH variation

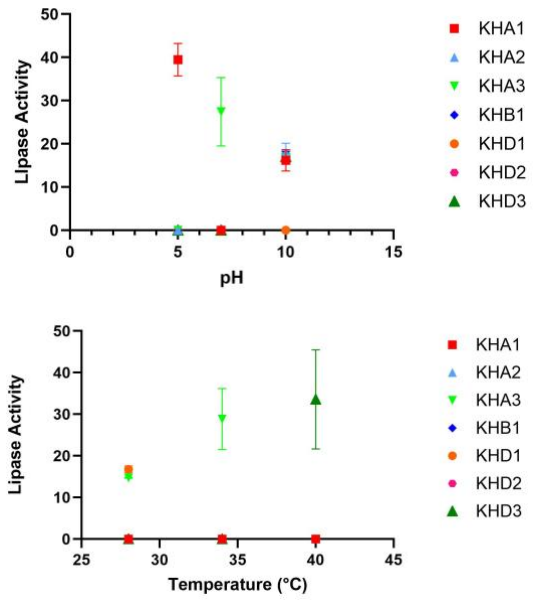

Figure 11. Lipase activity of endophytic bacteria of *K. hospita* L. under pH and temperature variation.

Although the largest opaque zone was recorded under acidic conditions in a single isolate, the higher number of lipase-producing isolates at pH 10 indicates that alkaline conditions were generally more favorable for lipase production. This trend is consistent with previous reports showing that bacterial lipases typically exhibit optimal activity under neutral to alkaline pH conditions due to enhanced enzyme stability and secretion [51]. The absence of lipase activity in some isolates under specific pH conditions may be attributed to differences in microbial physiology, substrate utilization, and growth phase. Lipase production is influenced by several factors, including microbial species, substrate availability, incubation time, and environmental conditions such as pH. The pH of the medium culture plays an important role in regulating microbial metabolism and enzyme synthesis. Optimal pH conditions support proper enzyme folding and catalytic activity, allowing microorganisms to efficiently produce and secrete lipase. In contrast, extreme pH can disrupt the ionization state of amino acid residues at the enzyme's active site, leading to structural changes that reduce catalytic efficiency or cause enzyme denaturation. In addition, unfavorable pH conditions may also affect microbial growth and cellular metabolism, thereby limiting the production and stability of lipase during fermentation [52].

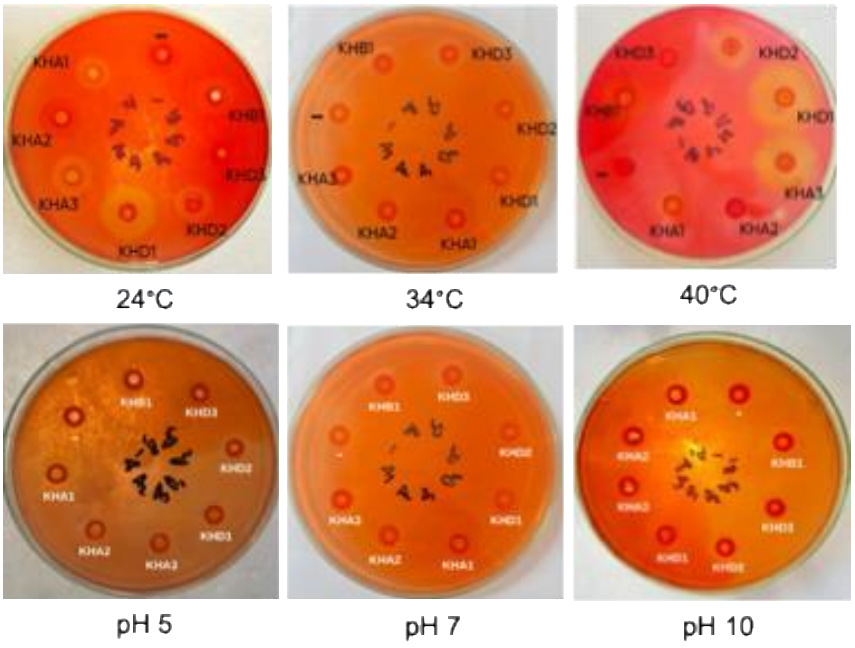

Figure 12. Clear zone diameter of cellulase activity produced by endophytic bacteria isolated from *K. hospita* L. under temperature and pH variation.

Fermentation temperature also significantly affected lipase activity. At 28 °C, lipase activity was detected only in isolates KHA3 and KHD2, whereas at 34 °C activity was observed exclusively in isolate KHA3. At 40 °C, lipase activity was detected only in isolate KHB1, which exhibited the largest opaque zone diameter (33.58 ± 11.93 mm). These results indicate that higher fermentation temperatures favored lipase production in certain isolates, although enzyme expression remained strongly isolate-dependent. Similar temperature-dependent patterns have been reported previously, with increased lipase production occurring at temperatures between 32 and 40 °C and optimal activity around 37 °C [53]. One-way ANOVA analysis indicated that lipase activity among the positive isolates did not differ significantly under different pH and temperature conditions ($p > 0.05$).

Cellulase enzyme activity was qualitatively detected by the formation of a light-yellow clear zone around microbial colonies grown on agar medium containing carboxymethyl cellulose (CMS), as shown in **Figure 12**. The effects of pH and fermentation temperature on cellulase activity of the endophytic bacterial isolates are summarized in **Figure 13**. Based on pH variation assays, cellulase activity was detected only in isolate KHA1 at pH 5, with a clear zone diameter of 18.13 ± 0.11 mm, while cellulase activity was not observed in other isolates or at other pH levels. These results indicate that cellulase production by the tested endophytic bacteria was favored under slightly acidic conditions. The present findings suggest that the cellulase produced by isolate KHA1 retained activity under mildly acidic conditions. Extreme acidic or alkaline pH conditions may disrupt enzyme conformation or inactivate essential catalytic residues, leading to reduced cellulase activity [23]. Fermentation temperature also exerted a significant influence on cellulase activity. At 28 °C and 40 °C, several isolates exhibited detectable cellulase activity, except for isolates KHA2 and KHD3, whereas cellulase activity was not observed at 34 °C. Among all tested samples, isolate KHD1 fermented at 40 °C exhibited the highest cellulase activity, as indicated by a clear zone diameter of 30.37 ±

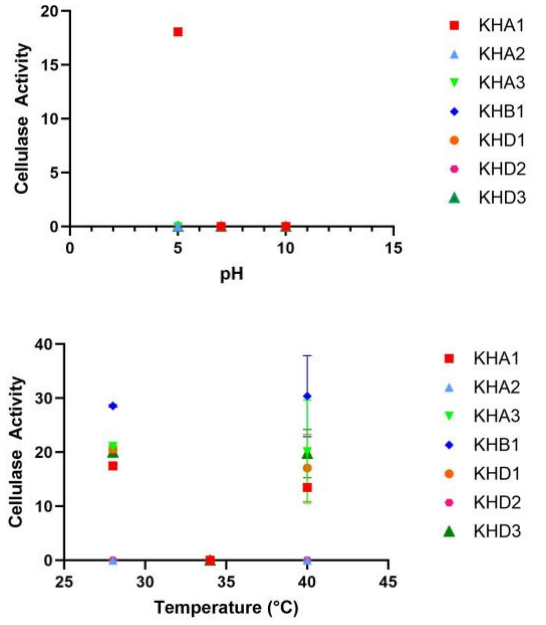

Figure 13. Cellulase activity of endophytic bacteria of *K. hospita* L. under pH and temperature

7.50 mm. Based on one-way ANOVA, variations in temperature did not significantly influence cellulase activity among the positive isolates ($p > 0.05$). These findings suggest that higher fermentation temperatures favored cellulase production in certain isolates. Previous studies have reported similar temperature-dependent patterns, with optimal endoglucanase activity in genera such as *Cellulomonas*, *Bacillus*, and *Micrococcus* occurring around 37–40 °C under near-neutral pH conditions. Generally, cellulase activity increases with temperature up to an optimum point and then declines due to thermal denaturation. The ability of cellulase enzymes to remain active at relatively elevated temperatures reflects enzymatic adaptation that enables microorganisms to maintain catalytic efficiency under fluctuating environmental conditions [54].

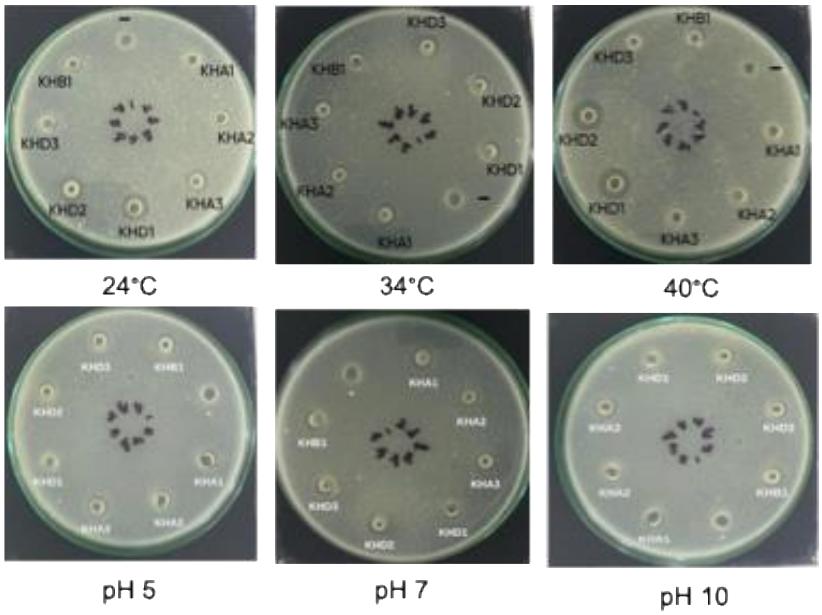

Figure 14. clear zone diameter of protease activity produced by endophytic bacteria isolated from *K. hospita* L. under temperature and pH variation.

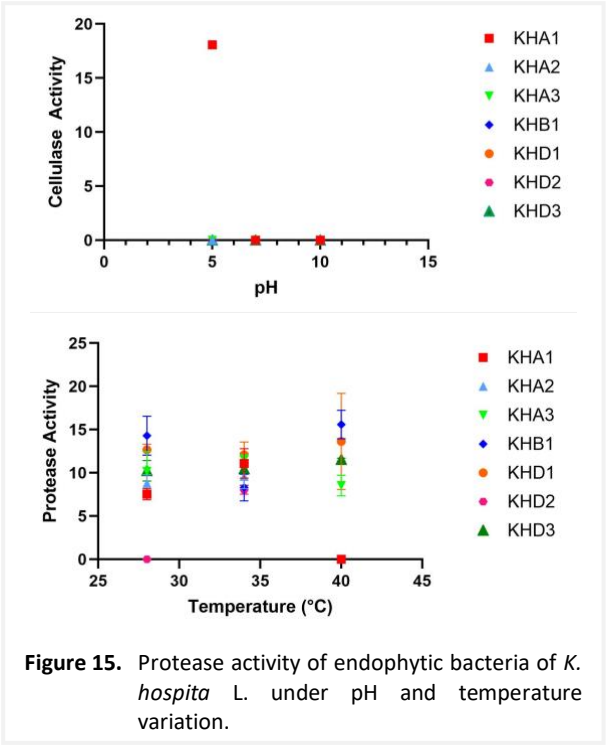

Figure 15. Protease activity of endophytic bacteria of *K. hospita* L. under pH and temperature variation.

The formation of a clear zone on solid medium, such as skim milk agar, indicates protease activity, where extracellular enzymes produced by microbes hy drolyze casein into clear, soluble peptides and amino acids, as shown in **Figure 14**. Protease activity was further analyzed under different pH and fermentation temperature conditions, as summarized in **Figure 15**. Based on pH variation assays, protease activity was detected in nearly all isolates. At pH values of 5 and 10, only isolate KHD1 did not exhibit detectable protease activity, whereas at pH 7 all isolates showed clear proteolytic activity, as indicated by the formation of transparent zones. The highest average clear zone diameter was recorded at pH 7 (12.09 ± 1.48 mm), indicating that neutral pH provided the most favorable condition for protease production by the endophytic bacterial isolates. This result is consistent with previous reports showing that bacterial proteases generally exhibit optimal activity at neutral pH, which promotes effective enzyme–substrate interactions and maximal formation of enzyme–substrate complexes [55]. Furthermore, the reported optimal pH range for the growth of proteolytic bacteria (pH 5–7) supports the observed protease activity pattern in this study. Protease production is known to be influenced by multiple physicochemical and nutritional factors, including

substrate availability, carbon and nitrog en sources, pH, temperature, dissolved oxygen, and inorganic salts [56]. Deviations from optimal pH conditions may alter enzyme conformation and reduce catalytic efficiency, thereby affecting both bacterial growth and extracellular protease secretion. Fermentation temperature also significantly affected protease activity among the isolates. At 28 °C, all isolates exhibited protease activity except KHD3, whereas at 40 °C, isolates KHA1, KHA2, and KHD3 showed no detectable activity. In contrast, at 34 °C, all isolates exhibited clear proteolytic activity, suggesting that moderate fermentation temperatures favor protease production. The diameter of the clear zone reflects the extent of protein hydrolysis and peptide bond cleavage by protease enzymes. Among all tested samples, the highest protease activity was observed in isolate KHD1 fermented at 40 °C, with a clear zone diameter of 15.35 ± 1.39 mm, indicating that certain isolates are capable of maintaining high protease activity at elevated temperatures. This observation is supported by previous studies reporting that the optimal temperature for free protease activity is approximately 45 °C, suggesting potential thermostability of proteases produced by specific isolates [57]. In addition, protein concentration in crude enzyme extracts has been reported to correlate positively with protease activity, where higher protein levels are often associated with increased enzymatic performance [58]. One-way ANOVA indicated a significant overall difference in protease activity under different ph and temperature conditions ($p < 0.05$), Tukey's post hoc test did not identify any statistically significant pairwise differences between isolates ($p > 0.05$), indicating that the observed variation among groups was relatively small and insufficient to distinguish specific differences among isolates.

Despite the promising results obtained in this study, several limitations should be acknowledged. The enzyme activity assays were primarily qualitative and based on the measurement of clear zone diameters on agar media, which provide only preliminary indications of enzymatic potential. Quantitative determination of enzyme activity, such as enzyme units or specific activity, was not performed. In addition, the enzymes produced by the endophytic bacterial isolates were not purified, and their biochemical properties, including optimal reaction conditions, substrate specificity, and kinetic parameters, were not characterized. Therefore, further studies involving enzyme purification, quantitative enzyme assays, and kinetic analyses are necessary to better understand the catalytic efficiency and potential industrial applicability of these enzymes.

CONCLUSION

Endophytic bacteria isolated from *K. hospita* L. exhibited varied extracellular enzymatic activities influenced by fermentation temperature, pH, and isolate characteristics. Statistical analysis indicated that some enzyme activities did not differ significantly under certain conditions, while others were interpreted descriptively due to limited detectable activity. In general, moderate temperatures and near-neutral pH tended to support higher protein production and enzyme activity, although optimal conditions varied among enzymes and isolates. Protease activity was widely detected, whereas pectinase and cellulase activities were limited to fewer isolates. Variations in enzymatic activity may be associated with differences in metabolic capacity and bacterial characteristics. These findings suggest that endophytic bacteria from *K. hospita* L. have

potential as sources of hydrolytic enzymes; however, further quantitative and biochemical characterization is required to confirm their applicability.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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REFERENCES

[1] Arifa, N., 2021, The Ethnobiological Society of Indonesia The Utilization of Tokulo (Kleinhovia hospita L .) as Traditional, J. Trop. Ethnobiol., IV (2), 105–110.

[2] Nagaraja, T. G., 2014, Seasonal occurrence of endomycophytes in inner bark of Kleinhovia hospital Linn., Nat. Environ. Pollut. Technol., 13 (1), 169–172.

[3] Drożdżyński, P., Rutkowska, N., Rodziewicz, M., and Marchut-Mikołajczyk, O., 2024, Bioactive Compounds Produced by Endophytic Bacteria and Their Plant Hosts—An Insight into the World of Chosen Herbaceous Ruderal Plants in Central Europe, Molecules, 29 (18), .

[4] Gouda, S., Das, G., Sen, S. K., Shin, H. S., and Patra, J. K., 2016, Endophytes: A treasure house of bioactive compounds of medicinal importance, Front. Microbiol., 7 (SEP), 1–8.

[5] Singh, M., Kumar, A., Singh, R., and Pandey, K. D., 2017, Endophytic bacteria: a new source of bioactive compounds, 3 Biotech, 7 (5), 1–14.

[6] Kandasamy, G. D., and Kathirvel, P., 2023, Insights into bacterial endophytic diversity and isolation with a focus on their potential applications –A review, Microbiol. Res., 266 (March 2022), 127256.

[7] Arung, E. T., Kusuma, I. W., Purwatiningsih, S., Roh, S. S., Yang, C. H., Jeon, S., Kim, Y. U., Sukaton, E., Susilo, J., Astuti, Y., Wicaksono, B. D., Sandra, F., Shimizu, K., and Kondo, R., 2009, Antioxidant Activity and Cytotoxicity of the Traditional Indonesian Medicine Tahongai (Kleinhovia hospita L.) Extract, JAMS J. Acupunct. Meridian Stud., 2 (4), 306–308.

[8] Yuliana, Y., and Herawati, S., 2016, Phytochemical Content and Protective Effect of Kleinhovia hospital Leaves Extract on Pancreatic Cytotoxicity in Hyperglycemic Rats (Kandungan Fitokimia Dan Efek Protektif Ekstrak Daun Paliasa (*Kleinhovia Hospita* Linn) Pada Sitotoksisitas Pankreas Tikus HI, J. Vet., 17 (3), 411–417.

[9] Masi, C., Tebiso, A., and Selva Kumar, K. V., 2023, Isolation and characterization of potential multiple extracellular enzyme-producing bacteria from waste dumping area in Addis Ababa, Heliyon, 9 (2), e12645.

[10] Cruz-Casas, D. E., Aguilar, C. N., Ascacio-Valdés, J. A., Rodríguez-Herrera, R., Chávez-González, M. L., and Flores-Gallegos, A. C., 2021, Enzymatic hydrolysis and microbial fermentation: The most favorable biotechnological methods for the release of bioactive peptides, Food Chem. Mol. Sci., 3, .

[11] Yousefi, N., Shokrollahi Yancheshmeh, B., and Gernaey, K. V., 2025, The Potential of Fermentation-Based Processing on Protein Modification: A Review, Foods, 14 (20), .

[12] Deepak, H., and Virk, V., 2022, Optimization of surface sterilization method for the isolation of endophytic fungi associated with Curcuma longa L. and their antibacterial activity, J. Adv. Biotechnol. Exp. Ther., 5 (2), 334–346.

[13] Tripathi, Nishant., Zubair, Muhammad., Sapra, A., 2025, Gram Staining, StatPearls Publishing LLC.

[14] Sharma, R., Garg, P., Kumar, P., Bhatia, S. K., and Kulshrestha, S., 2020, Microbial fermentation and its role in quality improvement of fermented foods, Fermentation, 6 (4), 1–20.

[15] Thermoscientific, 2017, Protein assay technical handbook, Thermo Fish. Sci., 1–60.

[16] Mettler-toledo, 2020, UV / VIS Application Note, Mettler Toledo, 1 (30355411), 1–2.

[17] Fatoni, A., and Zusfahair, 2012, Thermophilic amylase from Thermus sp. isolation and its potential application for bioethanol production, Songklanakarin J. Sci. Technol., 34 (5), 525–531.

[18] Msarah, M. J., Ibrahim, I., Hamid, A. A., and Aqma, W. S., 2020, Optimisation and production of alpha amylase from thermophilic Bacillus spp. and its application in food waste biodegradation, Heliyon, 6 (6), e04183.

[19] Inamdar, N., Dike, A., and Jadhav, A., 2024, Isolation, Screening, and Identification of Pectin Degrading Bacteria from Soil, J. Chem. Heal. Risks, 14 (2), 930–938.

[20] Mohandas, A., Raveendran, S., Parameswaran, B., Abraham, A., Athira, R. S. R., Mathew, A. K., and Pandey, A., 2018, Production of pectinase from bacillus sonorensis MPTD1, Food Technol. Biotechnol., 56 (1), 110–116.

[21] Kumar, D., Kumar, L., Nagar, S., Raina, C., Parshad, R., and Gupta, V. K., 2012, Screening, isolation and production of lipase/esterase producing Bacillus sp. strain DVL2 and its potential evaluation in esterification and resolution reactions, Arch. Appl. Sci. Res., 4 (4), 1763–1770.

[22] Ramnath, L., Sithole, B., and Govinden, R., 2017, Identification of lipolytic enzymes isolated from bacteria indigenous to Eucalyptus wood species for application in the pulping industry, Biotechnol. Reports, 15 (July), 114–124.

[23] Olivier, D., Omwenga, G. I., Cheruiyot, D. K., and Ngugi, M. P., 2026, Optimization of cellulase production by Nigrospora oryzae (berk and br.) petch and its application in biomass saccharification and ethanol production, Sci. African, 31 (January), e03181.

[24] Raveschot ab, C., Cudennec, B., Deracinois, B., Frémont, M., Dugersuren, J., Demberel, S., Drider, D., Coutte, F., and Flahaut, C., 2019, Proteolytic activity of Lactobacillus strains isolated from Mongolian traditional dairy products: a multiparametric analysis 2 3 Keywords Cell envelope associated peptidases, fermented milk, Lactobacillus, peptides,.

[25] Lin, H., Liu, C., Peng, Z., Tan, B., Wang, K., and Liu, Z., 2022, Distribution pattern of endophytic bacteria and fungi in tea plants, Front. Microbiol., 13 (September), .

[26] Mueller, C. W., Baumert, V., Carminati, A., Germon, A., Holz, M., Kögel-Knabner, I., Peth, S., Schlüter, S., Uteau, D., Vetterlein, D., Teixeira, P., and Vidal, A., 2024, From rhizosphere to detritusphere – Soil structure formation driven by plant roots and the interactions with soil biota, Soil Biol. Biochem., 193 (February), .

[27] Dogan, G., and Taskin, B., 2021, Hydrolytic enzymes producing bacterial endophytes of some poaceae plants, Polish J. Microbiol., 70 (3), 297–304.

[28] Tang, B., Lai, P., Weng, M., Wu, L., and Li, Y., 2022, Optimization of submerged fermentation conditions for biosynthesis of ergothioneine and enrichment of selenium from Pleurotus eryngii 528, Food Sci. Technol., 42.

[29] Tavarideh, F., Pourahmad, F., and Nemati, M., 2022, Diversity and antibacterial activity of endophytic bacteria associated with medicinal plant, Scrophularia striata, Vet. Res. Forum, 13 (3), 409–415.

[30] Ek-Ramos, M. J., Gomez-Flores, R., Orozco-Flores, A. A., Rodríguez-Padilla, C., González-Ochoa, G., and Tamez-Guerra, P., 2019, Bioactive products from plant-endophytic Gram-positive bacteria, Front. Microbiol., 10 (MAR), 1–12.

[31] Jain, B. P., Goswami, S. K., and Pandey, S., 2021, Protein, in Protocols in Biochemistry and Clinical Biochemistry, pp. 31–48.

[32] NR, S., Manish, M., and HB, S., 2020, Development of newer validated HPLC method for the determination of Alvimopan in Rat plasma, Int. J. Pharm. Biomed. Eng., 7 (3), 4–14.

[33] Fan, K. T., Xu, Y., and Hegeman, A. D., 2024, Elevated Temperature Effects on Protein Turnover Dynamics in Arabidopsis thaliana Seedlings Revealed by 15N-Stable Isotope Labeling and ProteinTurnover Algorithm, Int. J. Mol. Sci., 25 (11), .

[34] Rault, A., Bouix, M., and Béal, C., 2009, Fermentation pH influences the physiological-state dynamics of Lactobacillus bulgaricus CFL1 during pH-controlled culture, Appl. Environ. Microbiol., 75 (13), 4374–4381.

[35] Huang, M., Bao, J., Hallström, B. M., Petranovic, D., and Nielsen, J., 2017, Efficient protein production by yeast requires global tuning of metabolism, Nat. Commun., 8 (1), .

[36] Sharif, S., Shah, A. H., Fariq, A., Jannat, S., Rasheed, S., and Yasmin, A., 2023, Optimization of amylase production using response surface methodology from newly isolated thermophilic bacteria, Heliyon, 9 (1), e12901.
[37] Kim, S. H., Kim, W. J., Ryu, J., Yerefu, Y., and Tesfaw, A., 2025, Amylase Production by the New Strains of Kocuria rosea and Micrococcus endophyticus Isolated from Soil in the Guassa Community Conservation Area, Fermentation, 11 (4), 1–19.
[38] Krishnan, A., Alias, Z., Convey, P., González-Aravena, M., Smykla, J., Rizman-Idid, M., and Alias, S. A., 2022, Temperature and pH Profiling of Extracellular Amylase from Antarctic and Arctic Soil Microfungi, Fermentation, 8 (11), 1–12.
[39] Kholikov, A., Vokhidov, K., Murtozoyev, A., Tóth, Z. S., Nagy, G. N., Vértessy, B. G., and Makhsumkhanov, A., 2025, Characterization of a Thermostable α-Amylase from Bacillus licheniformis 104.K for Industrial Applications, Microorganisms, 13 (8), 1–14.
[40] Ren, H., Wang, T., and Liu, R., 2024, Correlation Analyses of Amylase and Protease Activities and Physicochemical Properties of Wheat Bran During Solid-State Fermentation, Foods, 13 (24), .
[41] Abd-Elhalem, B. T., El-Sawy, M., Gamal, R. F., and Abou-Taleb, K. A., 2015, Production of amylases from Bacillus amyloliquefaciens under submerged fermentation using some agro-industrial by-products, Ann. Agric. Sci., 60 (2), 193–202.
[42] Isabel, J. B., and Premalatha, M., 2025, Harnessing amylase producing novel bacterial strains from industrial waste: A bioprocessing and optimization approach, Int. J. Biol. Macromol., 332, .
[43] Jalil, M. T. M., and Ibrahim, D., 2021, Partial Purification and Characterisation of Pectinase Produced by Aspergillus niger LFP-1 Grown on Pomelo Peels as a Substrate. DOI : https://doi.org/10.21315/t, Trop. Lifer Sci. Res., 32 (1), 1–22.
[44] Chandel, V., Biswas, D., Roy, S., Vaidya, D., Verma, A., and Gupta, A., 2022, Current Advancements in Pectin: Extraction, Properties and Multifunctional Applications, Foods, 11 (17), 1–30.
[45] Shrestha, S., Chio, C., Khatiwada, J. R., Mokale Kognou, A. L., Chen, X., and Qin, W., 2023, Optimization of Cultural Conditions for Pectinase Production by Streptomyces sp. and Characterization of Partially Purified Enzymes, Microb. Physiol., 33 (1), 12–26.
[46] Vajanapanich, P., Nearmnala, P., Parkbhorn, J., Nutho, B., Rungrotmongkol, T., and Hongdilokkul, N., 2025, Catalytic Residue Reprogramming Enhances Enzyme Activity at Alkaline pH via Phenolate-Mediated Proton Transfer, ACS Synth. Biol., 14 (9), 3612–3623.
[47] Singh, L., Sharma, G., Sharma, A., Awasthi, G., Kumar, L., Ali, M. I., and Moin, S., 2020, Purification, Isolation, and Characterization of Esterase from Rhodococcus sp. lKe-021, J. Pure Appl. Microbiol., 14 (2), 1387–1395.
[48] Li, X., and Yu, H., 2013, Purification and characterization of an extracellular esterase with organic solvent tolerance from a halotolerant isolate, Salimicrobium sp. LY19, BMC Biotechnol., 13 (108), .
[49] Mussakhmetov, A., and Silayev, D., 2025, Esterases: Mechanisms of Action, Biological Functions, and
[50] Frąc, M., Kaczmarek, J., and Jędryczka, M., 2022, Metabolic Capacity Differentiates Plenodomus lingam from P. biglobosus Subclade 'brassicae', the Causal Agents of Phoma Leaf Spotting and Stem Canker of Oilseed Rape (Brassica napus) in Agricultural Ecosystems, Pathogens, 11 (1), .
[51] Ali, S., Khan, S. A., Hamayun, M., and Lee, I. J., 2023, The Recent Advances in the Utility of Microbial Lipases: A Review, Microorganisms, 11 (2), .
[52] Abdelaziz, A. A., Abo-Kamar, A. M., Elkotb, E. S., and Al-Madboly, L. A., 2025, Microbial lipases: advances in production, purification, biochemical characterization, and multifaceted applications in industry and medicine, Microb. Cell Fact., 24 (1), .
[53] Colla, L. M., Ficanha, A. M. M., Rizzardi, J., Bertolin, T. E., Reinehr, C. O., and Costa, J. A. V., 2015, Production and characterization of lipases by two new isolates of Aspergillus through solid-state and submerged fermentation, Biomed Res. Int., 2015, .
[54] Lynd, L. R., Weimer, P. J., Zyl, W. H. Van, and Isak, S., 2002, Microbial Cellulose Utilization : Fundamentals and Biotechnology Microbial Cellulose Utilization : Fundamentals and Biotechnology Downloaded from http://mmbr.asm.org/ on February 6 , 2013 by Indian Institute Of Technology Madras, Microbiol. Mol. Biol. Rev., 66 (3), 506–577.
[55] Song, P., Zhang, X., Wang, S., Xu, W., Wang, F., Fu, R., and Wei, F., 2023, Microbial proteases and their applications, Front. Microbiol., 14 (September), 1–24.
[56] Abusham, R. A., Rahman, R. N. Z. R. A., Salleh, A., and Basri, M., 2009, Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant Bacillus subtilis strain Rand, Microb. Cell Fact., 8, 1–9.
[57] Gomes, J. E. G., Rosa, I. Z., Nascimento, T. C. E. da S., Souza-Motta, C. M. de, Gomes, E., Boscolo, M., Moreira, K. A., Pintado, M. M. E., and da Silva, R., 2020, Biochemical and thermodynamic characteristics of a new serine protease from Mucor subtilissimus URM 4133, Biotechnol. Reports, 28, .
[58] Sharma, A., Sharma, S., Ramaraju, G., Rasane, P., Ercisli, S., and Singh, J., 2025, Enzyme-assisted extraction of leaf proteins: efficiency, functionality, and structural insights, Food Chem. X, 31 (February), 103181.