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Production of Protease Enzymes and Bioactive Compounds by Lactic Acid Bacteria (LAB) From Broccoli (*Brassica oleracea* L.) Using *Lactobacillus plantarum* Starter

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Abstract: This study aimed to isolate and characterize Lactic Acid Bacteria (LAB) from broccoli (*Brassica oleracea* L.) with potential protease-producing activity and probiotic properties. Fermentation was carried out using *Lactobacillus plantarum* as a starter culture. The obtained LAB isolates were subsequently evaluated for protease activity and analyzed for bioactive compound content. The results demonstrated that LAB isolates obtained from broccoli were capable of producing proteases with significant activity, indicating their potential for biotechnological applications. In addition, the isolates produced bioactive compounds exhibiting antibacterial activity against several pathogenic bacteria, including *S. epidermidis*, *K. pneumoniae*, *E. coli*, *S. aureus*, *S. typhi*, and *S. pyogenes,* as evidenced by the formation of inhibition zones. The isolate demonstrated strong antibacterial activity against all tested pathogens with inhibition zones of 56 mm for *S. epidermidis*, 30 mm for *S. typhi*, 12 mm for both *K. pneumoniae* and *E. coli,* and 11 mm for *S. pyogenes* and *S. aureus*. Acid tolerance assays demonstrated that the LAB isolates survived at low pH, and protease activity assays showed that the isolate produced an enzyme with proteolytic activity of 23.5 mm and 0.0440 U/mL.

Keywords: Broccoli, Protease, Lactic Acid, Probiotic

Introduction

Broccoli *(Brassica oleracea* L.*)* is recognized as a rich source of exogenous antioxidants with high antioxidant activity. The bioactive compounds present in broccoli include flavonoids, hydroxycinnamic acids, glucosinolates, polyphenols, and sulforaphane. Flavonoids stabilize free radicals by donating hydrogen atoms, thereby preventing oxidative damage to pancreatic β-cells and preserving insulin function. In addition, sulforaphane acts as a potent antioxidant and antidiabetic compound, contributing to reducing blood glucose levels [1]. Broccoli is rich in minerals such as sulfur, potassium, calcium, iron, phosphorus, and folic acid, as well as various B vitamins, vitamin C, and beta carotene. Beyond its anticancer compounds, including vitamin C, beta-carotene, and dietary fiber, broccoli contains numerous phytochemicals that provide protective effects against cancer and cardiovascular diseases. Its high fiber content also helps manage diabetes [2].

Various plant extracts contain bioactive compounds that inhibit the growth of pathogenic bacteria. Antibacterial agents generally exhibit stronger bactericidal than

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bacteriostatic effects. They can be categorized as antibiotics, antivirals, antiparasitics, or antifungals and further classified based on their spectrum of activity, mechanism of action, producing organism, biosynthetic pathway, and chemical structure [3]. The antibacterial mechanism involves inhibiting cell wall synthesis, increasing cell membrane permeability, and disrupting protein synthesis, ultimately leading to bacterial growth inhibition or cell death. Antibacterial activity is commonly evaluated using the agar diffusion method, which is practical, rapid, and allows precise control of test-substance concentration, making it a preferred approach [4].

LAB produce secondary metabolites, including hydroxy fatty acids, diacetyl, and reuterin, as well as proteinaceous compounds such as bacteriocins, trypsin, and peptides, which contribute to the inhibition of pathogenic bacterial growth [5]. LAB is considered safe for consumption and is classified as GRAS (Generally Recognized As Safe). Certain LAB strains produce antibacterial compounds that play a crucial role in the food industry, particularly in food preservation and shelf life extension [6]. Consequently, it is important to isolate and characterize the bioactive compounds of LAB from broccoli, with a focus on their potential as enzyme producers and probiotic agents, using Lactobacillus plantarum as a starter culture.

Materials and Methods

Materials

The materials used in this research are Broccoli (*Brassica oleracea* L.), *Lactobacillus plantarum*, distilled water, 37% hydrochloric acid (HCl), sodium hydroxide (NaOH), nutrient agar, nutrient broth, Mueller Hilton agar, crystal violet, Lugol, alcohol, safranin, *Staphylococcus epidermidis, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Salmonella typhi, Streptococcus pyogenes*, disc paper, erythromycin antibiotic, 95% ethanol, silica gel GF-254, Mg powder, concentrated hydrochloric acid (HCl), phenol follin (reagent D), (reagent C), 0.05 M phosphate buffer, 2% casein, crude enzyme, 0.1 M TCA, 2% hydrochloric acid (HCl), Wigner's reagent, 5% $FeCl_3$, anhydrous acid, concentrated H_2SO_4, DNS reagent, 1% starch, 70% alcohol, methylated spirits, aluminum foil, plastic wrap.

Broccoli Fermentation Using *Lactobacillus plantarum* Starter

One gram of broccoli was placed into a prepared liquid medium, followed by the addition of 1 mL of *Lactobacillus plantarum* starter culture. The mixture was sealed with aluminium foil and plastic wrap to maintain anaerobic conditions and incubated at 37 °C for 24 h. The pH of the solution was recorded before and after fermentation. Subsequently, the isolate was cultured on solid nutrient agar and incubated at 37 °C for an additional 24 h.

pH Resistance Test

LAB exhibits a wide pH tolerance range. Acid resistance was evaluated at pH 1.0, 2.0, 3.0, 4.0, and 5.0. Bacteria isolates were inoculated into Nutrient Broth (NB) and

adjusted to the desired pH using 37% HCl for acidic conditions and NaOH for alkaline conditions [7].

Gram Staining Test

Gram staining was performed by applying 1 drop of crystal violet to a smear of the LAB isolate, allowing it to stand for 1 minute, and then rinsing with distilled water. One drop of iodine was then added, left for 1 minute, and rinsed with running water. The smear was decolorized with 95% ethanol, rinsed again with running water, and then counterstained with a drop of safranin. Gram-positive bacteria retain the crystal violet dye, appearing purple, whereas Gram-negative bacteria don't retain it and appear red after safranin staining [8,9]. The stained preparations were air-dried and observed under a microscope.

Antimicrobial Activity Test

Antimicrobial activity was tested using Mueller-Hinton Agar (MHA). A total of 3.8 g MHA was dissolved in 100 mL of distilled water, and 15 mL of medium was poured into each of six petri dishes. Pathogenic bacteria, consisting of *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, and *Streptococcus pyogenes,* were evenly spread over the agar surface. Erythromycin antibiotic disks were placed on the medium as a positive control. The plates were incubated at 37 °C for 24 h, and the resulting zones of inhibition were measured using a ruler [10].

Protease Enzyme Activity Test

Before the enzyme activity assay, the pure broccoli isolate was rejuvenated by streaking one loopful into agar medium and incubating at 37 °C for 24 h. The rejuvenated culture was then transferred into liquid broth and incubated at 37 °C for an additional 24 h. The resulting bacterial isolates were used to assess protease activity through protease enzyme screening and quantification using a UV-Vis spectrophotometer.

Results and Discussions

Broccoli Fermentation Using *Lactobacillus plantarum* Starter

Broccoli fermentation involves the conversion of natural sugars in broccoli into lactic acid by microorganisms such as *Lactobacillus spp*., *Leuconostoc* spp., and *Pediococcus* spp. This process not only extends broccoli's shelf life but also enhances its nutritional value by introducing probiotics that support digestive health. Additionally, fermentation produces bioactive compounds, including sulforaphane, which is recognized for its anticancer properties [11]. LAB are microorganisms that primarily produce lactic acid through carbohydrate fermentation. They are widely used in food fermentation processes due to their ability to inhibit the growth of pathogenic microorganisms by producing organic acids and antimicrobial compounds [12].

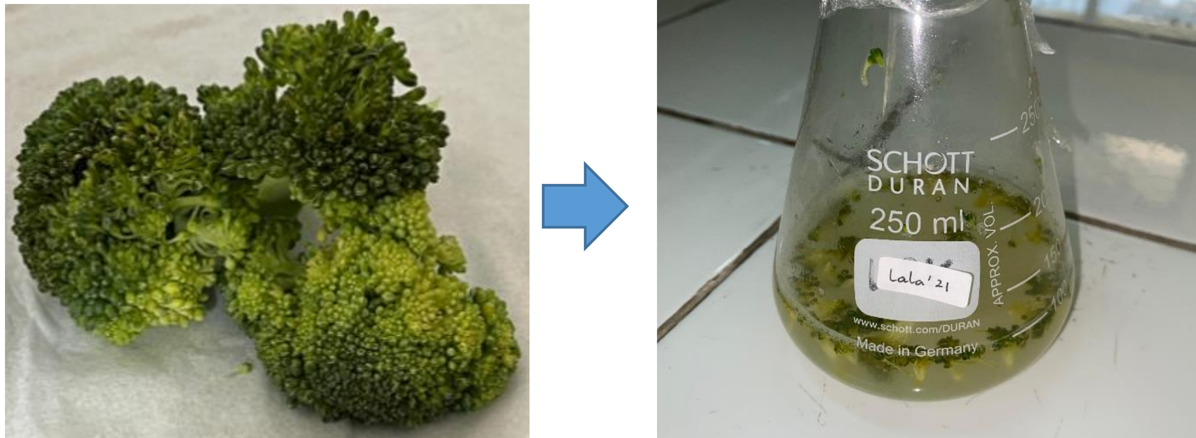

Figure 1. Broccoli fermentation process using *L. plantarum*

L. plantarum is a lactic acid bacterium commonly employed in the fermentation of vegetables, including broccoli. Fermentation with *L. plantarum* produces lactic acid, prolonging the shelf life of broccoli and enhancing its probiotic content, thereby promoting digestive health [13]. Additionally, this fermentation increases the levels of bioactive compounds such as sulforaphane, known for its anticancer properties and antioxidant activity [11]. Overall, *L. plantarum* in fermented broccoli has significant potential to enhance nutritional value and health benefits through the production of probiotics and antioxidant compounds that support overall well-being. Fermentation is conducted under anaerobic conditions, which are essential for the optimal growth of *L. plantarum*. The fermentation reaction can be summarized as follows:

$$C_6H_{12}O_6 + H_2O \rightarrow 2CH_3CHOHCOOH + CH_3COOH + C_2H_5OH + 2CO_2 + 2H_2$$

Glucose   Lactic acid   Acetic acid   Ethyl alcohol

L. plantarum ferments the carbohydrates in broccoli to produce lactic acid. The accumulation of lactic acid lowers the pH, creating an acidic environment during fermentation. This acidic condition inhibits the growth of pathogenic and spoilage microorganisms that are sensitive to low pH.

Acid Resistance Test

Acid resistance testing of LAB, such as *L. plantarum,* is conducted to assess their ability to survive in acidic environments. This assessment is crucial, as probiotic bacteria in fermented foods must withstand the acidic environment of the stomach to reach the intestine and confer health benefits [14]. Typically, this test involves cultivating LAB in a medium adjusted to a low pH 2-4 value. This setup closely simulates the conditions of the human stomach. LAB capable of surviving low pH levels are considered potential probiotics as they can endure passage through the digestive tract [15]. The acid resistance test is conducted by adjusting the medium with 37% HCl to create an acidic environment and 10% NaOH to establish an alkaline environment (**Table 1**).

Table 1. The acid resistance test of LAB: Growth rate for 24 h

pH	Growth rate
1.0	+
2.0	+
3.0	+
4.0	++
5.0	+++

Note: + Slightly; ++ cloudy; +++ very cloudy

The results in **Table 1** indicate that *L. plantarum* isolates exhibit optimal growth at acidic pH values, particularly at pH 4.0 and 5.0. Growth was limited at pH 1.0, 2.0, and 3.0, suggesting that *L. plantarum* demonstrates strong acid tolerance at pH 4.0, which approximates the pH of the human digestive tract. Additionally, LAB grew well in NB medium with near-neutral pH, as the medium typically has a pH between 5.0 and 6.0.

L. plantarum exhibited poor growth at pH 1.0, 2.0, and 3.0, as highly acidic conditions can compromise bacterial cell structure and function. Under such conditions, the cell wall and plasma membrane may be disrupted, leading to loss of cell integrity and impairment of essential metabolic processes, including protein synthesis and DNA replication. Additionally, extreme acidity generates an excessive proton gradient across the cell membrane, inducing acid stress that hinders the bacteria's ability to maintain intracellular pH homeostasis required for survival [16].

Gram Staining Test

The Gram staining test differentiates bacteria based on cell wall structure, specifically the thickness of the peptidoglycan layer. Gram-positive bacteria possess a thick peptidoglycan layer that retains the crystal violet dye after decolorization with alcohol or acetate. In contrast, Gram-negative bacteria have a thinner peptidoglycan layer and a more complex outer membrane, which causes them to lose the crystal violet stain and subsequently take up safranin, resulting in pink coloration [14]. The reagents used in the Gram stain test include crystal violet, Lugol's solution, and safranin, with alcohol or acetate as a decolorizer. Crystal violet stains all bacterial cells, while Lugol's solution serves as a mordant, forming a complex with the dye and binding it to the cell wall. Alcohol or acetate decolorizes Gram-negative bacteria, which are then counterstained with safranin, resulting in a pink coloration.

To perform Gram staining, a bacterial smear is first prepared on a glass slide and then gently heat-fixed. A drop of crystal violet is applied, allowed to stand for 1 minute, then rinsed with water. Lugol's solution is then added as a mordant for 1 minute, and the sample is rinsed. The smear is colorized with alcohol or acetate until Gram-negative cells lose their purple color. Safranin is subsequently applied for 1 minute as a counterstain, followed by a final rinse and air drying. The slide is then examined under a microscope.

A purple-stained isolate is identified as Gram-positive. Gram staining of the isolate revealed a purple coloration with a cylindrical or bacillus morphology. The bacteria were observed as single cells or in short chains, indicating that the isolate belongs to the LAB group.

The Gram-positive, rod-shaped, non-spore-forming morphology is characteristic of several LAB species, including *L. plantarum*, *L. acidophilus*, and *L. delbrueckii*. Rod-shaped LAB are commonly associated with fermentation processes where they convert carbohydrates into lactic acid. These bacteria play a crucial role in the production of fermented foods and beverages such as yogurt and sauerkraut. Notably, *L. plantarum* is a rod-shaped LAB commonly present in the human gastrointestinal tract and is widely utilized as a probiotic [18].

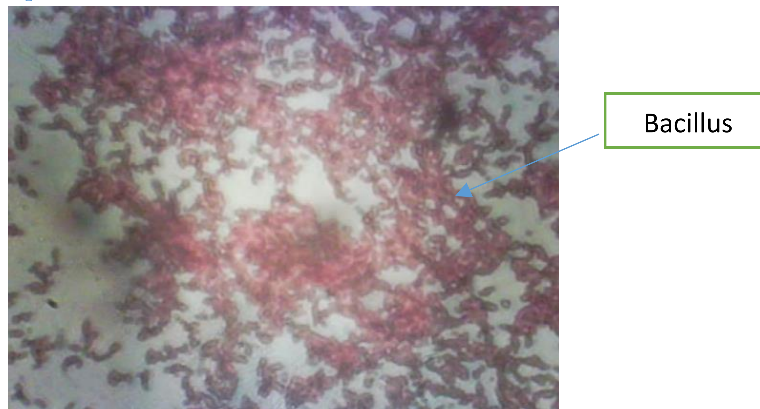

Figure 2. Gram staining of a broccoli sample

Antimicrobial Activity Test

Antimicrobial activity was evaluated using the disc diffusion method. Sterile paper discs impregnated with extracts or antimicrobial solutions were placed on the surface of agar inoculated with the test microorganisms. Discs were positioned using sterile tweezers to maintain stability and ensure even diffusion of the compounds into the medium. A positive control (erythromycin) was also applied at a designated distance to facilitate comparison. Erythromycin primarily exhibits bacteriostatic activity by inhibiting bacterial growth, although it can be bactericidal at higher concentrations or against specific bacterial strains.

The petri dishes were covered and incubated at 37 °C for 24 h. During incubation, antimicrobial compounds diffused into the agar, establishing a concentration gradient around each disc. Compounds exhibiting antimicrobial activity produced a clear zone of inhibition surrounding the disc, representing the area where microbial growth was suppressed. The diameter of the inhibition zone was measured to assess the antimicrobial potency of the test sample, with larger zones indicating stronger activity [19].

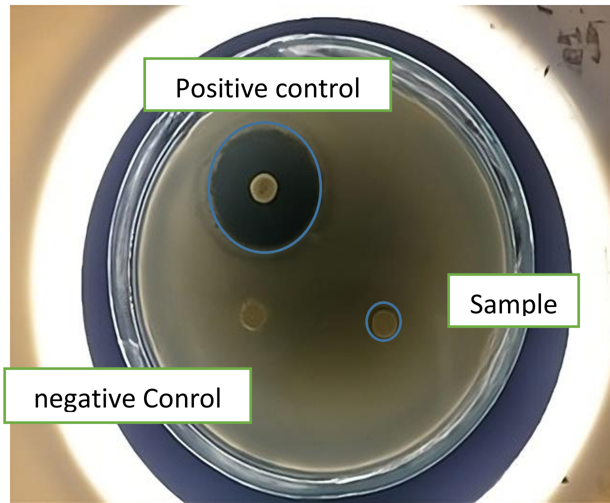

Figure 3. Antimicrobial activity test result for *S. epidemidis* for 24 h

Bacteria are classified as sensitive when the zone of inhibition is sufficiently large, indicating that the antibiotic is effective at standard therapeutic doses. An intermediate classification is assigned when the inhibition zone falls between the sensitive and resistant threshold, suggesting that the antibiotic may be effective at higher doses or if concentrated at the site of infection [20]. Bacteria are considered resistant when the inhibition zone is small or absent, indicating that the antibiotic is ineffective at inhibiting or killing the bacteria at concentrations safe for human use [21].

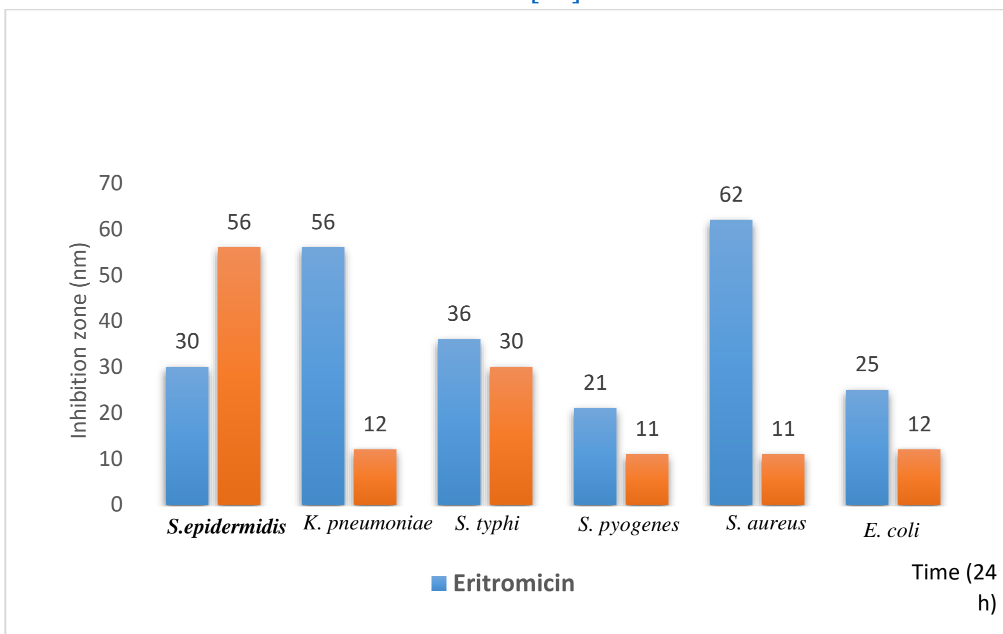

Figure 4. Inhibition zone of test bacteria for 24 h

Based on the graph, the isolate exhibited the highest antimicrobial activity against *S. epidermidis* with a clear zone diameter of approximately 56 mm after 24 h. The activity against *S. typhi* resulted in a clear zone of approximately 30 mm. The inhibition zones for *K. pneumoniae* and *E. coli* were about 12 mm, while those for *S. pyogenes* and *S. aureus* were approximately 11 mm each. These results indicate that the isolate shows varying

antimicrobial activity against different pathogens, with notably strong inhibition of *S. epidermidis* and moderate inhibition of *S. typhi*.

Protease Enzyme Activity Test

Quantitative assessment of protease activity is commonly performed to evaluate the enzyme's ability to hydrolyze protein substrates into peptides or free amino acids. A widely used approach is the spectrophotometric method employing casein as the substrate. Protease hydrolyzes casein into soluble peptides, which are subsequently precipitated with an acid solution such as TCA. The concentration of soluble peptides or released tyrosine is then measured spectrophotometrically, typically at 280 nm. The casein-based spectrophotometric method remains a standard for determining protease activity, with modifications introduced in recent studies to enhance sensitivity and accuracy [22].

Hydrolysis of peptide bonds in proteins or peptides involves cleavage of amide bonds (-CO-NH-) between amino acid residues, generating smaller molecules such as oligopeptides, short peptides, or free amino acids. The protease enzyme showed an activity of 23.5 mm after 24 h of incubation. The formation of a clear zone in a solid medium indicates protease activity. Bacteria can secrete a protease enzyme to degrade proteins in the medium. This process alters the structure, function, and chemical properties of the hydrolyzed protein [23]. Peptide hydrolysis can produce bioactive compounds with potential applications in managing metabolic disorders, including hypertension and diabetes [24], and enhances the bioavailability of proteins and amino acids in functional food formulations [25].

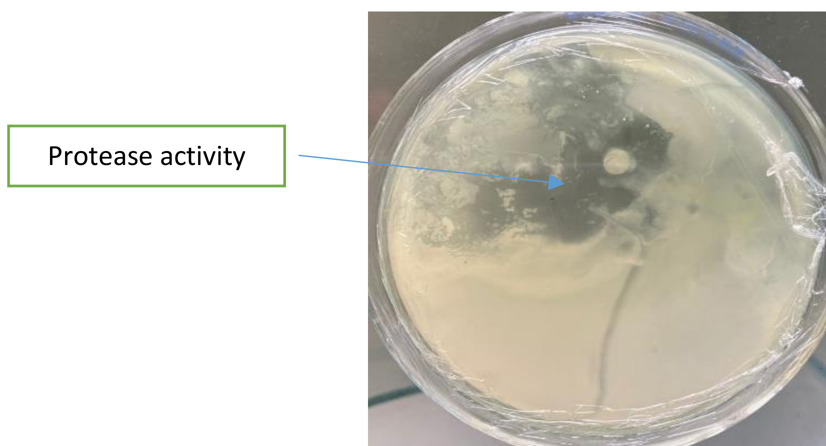

Figure 5. Screening protease enzyme activity for 24 h

The BSA standard curve was used to determine protein concentration by comparing the sample absorbance with that of BSA standard solutions at 578 nm. The absorbance values were plotted against concentration to generate a regression equation: Y= 0.0001703x – 0.001571429. Using this equation, the protein concentration of the sample was calculated as 2.4396 mg/mL. Protease enzyme activity was measured at 0.0440 U/mL with a specific activity of 0.0180 U/mg. The relatively low specific activity is attributed to the crude extract, which contains non-enzymatic proteins that reduce enzyme

efficiency. Nevertheless, this value is reasonable, as the enzyme has not undergone purification, a process that typically increases its concentration and activity.

Conclusion

Identification was performed based on morphological characteristics, Gram staining, and biochemical tests. Gram staining revealed that the isolate is a Gram-positive, rod-shaped bacterium, confirming its classification within the LAB group. The isolate exhibited strong antibacterial activity against several pathogenic bacteria, including *S. epidermidis* (56 mm), *K. pneumoniae* (12 mm), *S. thyphi* (30 mm), *S. pyogenes* (11 mm), *S. aureus* (11 mm), *and E. coli* (12 mm). The isolate demonstrated high tolerance to low pH (4-5), indicating its potential to survive gastrointestinal conditions, maintain intestinal microbial balance, enhance immunity, and support digestive health. Protease activity assays showed that the isolate produces an enzyme with proteolytic activity of 23.5 mm and 0.0440 U/mL.

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