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Issue Article

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SELECTION OF STRAINS WITH HIGH TANNASE AND ANTIOXIDANT ACTIVITY FOR THE PRODUCTION OF FUNCTIONAL FOODS

Abstract: *This study presents the results of isolating bacterial strains with tannase activity from locally fermented and pickled food products and evaluating their antioxidant properties. Using MALDI-TOF MS analysis, the taxonomic identity of 17 isolated strains was determined. Screening revealed high tannase activity in Microbacterium sp., Paenibacillus amylolyticus, Pediococcus acidilactici, and Enterobacter cloacae. A major novelty of this work is the first report of tannic acid degradation by P. amylolyticus and P. acidilactici. In addition, DPPH radical scavenging assays confirmed a high antioxidant potential of the selected strains, ranging from 65.2% to 72.8%. These strains are considered promising candidates for ensuring food safety, reducing antinutritional factors, and for applications in the food and pharmaceutical industries.*

Key words: *lactic acid bacteria, tannase, tannic acid, DPPH, Pediococcus acidilactici, biotransformation, gallic acid.*

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Introduction

Distribution and biological significance of tannins

Tannins are high-molecular-weight polyphenolic compounds that rank third in abundance among plant constituents after cellulose and lignin [1,2]. They accumulate in various plant parts, including stems, leaves, fruits, and bark, where they play a protective role against insects, pathogenic microorganisms, and ultraviolet radiation. Based on their chemical structure, tannins are mainly classified into hydrolysable tannins (e.g., gallotannins) and condensed tannins (proanthocyanidins) [3].

Antinutritional properties and environmental concerns

Despite their antioxidant capacity, tannins are often classified as antinutritional compounds. They form stable, insoluble complexes with proteins, polysaccharides, and minerals, thereby reducing the activity of digestive enzymes such as trypsin, chymotrypsin, and amylase and significantly impairing protein bioavailability. Moreover, tannin-rich wastewater generated by the leather and textile industries poses serious environmental challenges, as tannins are poorly biodegradable and may be toxic to microorganisms [4].

Tannase and its industrial relevance

Tannase (tannin acyl hydrolase, EC 3.1.1.20) plays a key role in tannin degradation by hydrolyzing ester and depside bonds in gallotannins, resulting in the formation of gallic acid and glucose. Gallic acid is a valuable raw material for the synthesis of pharmaceuticals such as trimethoprim and food antioxidants such as propyl gallate [5].

Lactic acid bacteria and tannase activity

In recent years, increasing attention has been paid to lactic acid bacteria (LAB), particularly members of the genera Lactobacillus and Lactiplantibacillus, as potential tannase producers. LAB are generally recognized as safe (GRAS) and possess probiotic properties. By degrading tannins, LAB reduce bitterness in fruits and vegetables and enhance their nutritional value. Furthermore, bacterial tannases may exhibit greater stability at higher pH values and temperatures compared to fungal tannases, making them more suitable for biotechnological applications [6,7,18].

Rationale of the study

Most industrial tannases are currently derived from fungi, although their production and purification processes are complex. The isolation of LAB strains with high tannase activity from locally fermented foods and the characterization of their biological and technological properties open new prospects for both bioremediation and functional food production.

The main objective of this study was to isolate indigenous LAB strains capable of efficiently degrading tannic acid from fermented and pickled foods, to assess their tannase activity, and to characterize strains with potential applications in the food and pharmaceutical industries.

2. MATERIALS AND METHODS

2.1. Isolation and purification of lactic acid bacteria

Microbiological sampling was performed using various locally produced fermented and pickled food products. All procedures were carried out in accordance with aseptic techniques to prevent external contamination. To ensure effective separation of bacterial cells from solid substrates (fruit and vegetable tissues), samples were homogenized and subjected to mechanical agitation using a vortex mixer for 10 seconds, as previously described [8].

Serial decimal dilutions of each homogenized sample were prepared in sterile physiological saline, ranging from 10^{-1} to 10^{-7}. From each dilution, 1 mL aliquots were plated onto selective de Man, Rogosa and Sharpe (MRS) agar using the surface-spreading technique. The inoculated plates were incubated at 37 ± 1 °C for 48 hours under microaerophilic conditions to promote the growth of lactic acid bacteria.

After incubation, morphologically distinct colonies were selected based on size, shape, color, surface texture, and margin characteristics. Selected colonies were repeatedly subcultured on fresh MRS agar plates until pure cultures were obtained. Microscopic examination and Gram staining were performed to confirm cellular morphology and Gram reaction.

For accurate taxonomic identification, purified isolates were analyzed using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Protein mass spectra obtained from each isolate were compared with reference spectra from the instrument's database, allowing reliable identification at the genus and species levels. For long-term preservation, isolates were stored in sterile MRS broth supplemented with 20% (v/v) glycerol at −20 °C or −80 °C, which is considered an appropriate and widely accepted storage method.

2.2. Qualitative determination of tannase activity

The ability of isolated strains to degrade tannic acid was evaluated using a tannin-agar plate assay. This method allows the visual detection of tannase activity through the formation of hydrolysis zones around bacterial colonies.

Preparation of tannin-containing substrate

Pomegranate (Punica granatum L.) peel was used as a natural source of tannins, as previous studies have reported that its dry matter contains approximately 4–8% tannins. Fresh peels were thoroughly washed with tap water followed by distilled water to remove surface contaminants and then air-dried under laboratory conditions [9].

Dried peels (10–20 g) were cut into small fragments and boiled in 200–500 mL of distilled water for 30–60 minutes at moderate heat. The resulting extract was cooled to room temperature and sterilized by filtration through a 0.22 µm membrane filter. To prevent oxidative degradation of tannins, the sterile extract was stored in dark conditions until further use.

Preparation of culture medium

A modified MRS agar medium was used for screening tannase-producing lactic acid bacteria. The medium composition (g/L) was as follows: peptone – 10.0; meat extract – 8.0; yeast extract – 4.0; glucose – 20.0; sodium acetate – 5.0; triammonium citrate – 2.0; K_2HPO_4 – 2.0; $MgSO_4 \cdot 7H_2O$ – 0.2; $MnSO_4 \cdot H_2O$ – 0.05; Tween 80 – 1.0 mL; agar – 15.0–20.0, with distilled water added to a final volume of 1 L. The pH

of the medium was adjusted to 6.2 ± 0.2 prior to sterilization.

The medium was autoclaved at 121 °C under 1 atm pressure for 30 minutes. After cooling to approximately 50 °C, 20 mL of sterile tannic acid suspension was aseptically added to the medium, which was then poured into Petri dishes [10]. Pure bacterial cultures were spot-inoculated onto the tannin-containing agar and incubated at 37 °C for 48–72 hours.

2.3. Visualization and evaluation of hydrolysis zones

To confirm tannase activity and detect tannin degradation products such as gallic acid, a ferric chloride ($FeCl_3$) assay was applied. After incubation, 1–2 mL of freshly prepared 0.01 M $FeCl_3$ solution (in 0.01 N HCl) was poured onto the surface of each agar plate. After a 10-minute exposure period, excess reagent was removed.

The formation of dark blue, green, or brown zones around bacterial colonies indicated the complexation of ferric ions with phenolic degradation products, thereby confirming tannic acid hydrolysis. Plain MRS agar without tannic acid was used as a negative control to enhance the reliability and scientific validity of the assay [11].

2.4. Determination of antioxidant activity of lactic acid bacteria

Prior to antioxidant analysis, all lactic acid bacterial strains were activated in MRS broth by incubation at 37 °C for 24 hours and subcultured at least three consecutive times to ensure metabolic stability. Following the third incubation, bacterial cultures were centrifuged at 15,000 rpm for 15 minutes, and the supernatants were collected for analysis.

Cell pellets were washed twice with sterile 0.9% NaCl solution and resuspended in 0.2 M phosphate buffer (pH 7.4). Cell density was adjusted to approximately 10^8 CFU/mL (6 log CFU) according to the McFarland standard. Ascorbic acid (vitamin C) at a concentration of 1 mg/mL was used as a positive control [12].

2.4.1. DPPH radical scavenging assay

The antioxidant activity of bacterial supernatants and cell suspensions was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. This method is based on the ability of antioxidant compounds to donate electrons or hydrogen atoms, resulting in the reduction of the DPPH radical and a visible color change from deep violet to pale yellow.

The assay was performed according to a partially modified method described by Li et al. [13]. Briefly, 1 mL of bacterial supernatant or cell suspension was mixed with 1 mL of freshly prepared DPPH solution (0.05 mM in ethanol). For blank samples, phosphate buffer was used instead of DPPH solution. The mixtures were thoroughly vortexed and incubated in the dark at room temperature for 60 minutes.

After incubation, samples were centrifuged at 13,000 rpm for 10 minutes, and the absorbance of the supernatant was measured at 517 nm using a spectrophotometer. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = [1 - (A_{sample} - A_{blind}) / A_{blank}] \times 100$$

Where; A_{blind} represents the absorbance of phosphate buffer,

A_{blank} represents the absorbance of the DPPH solution,

and A_{sample} represents the absorbance of the sample-containing DPPH solution.

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3. RESULTS AND DISCUSSION

3.1. Results of microbiological isolation and taxonomic identification

Within the scope of the present study, microbiological samples were collected from a total of ten different locally produced fermented and pickled food sources. Using selective cultivation on MRS agar and repeated subculturing, 17 pure microbial isolates were successfully obtained.

At the preliminary stage, all isolates were subjected to phenotypic characterization, including the evaluation of colony morphology (shape, color, surface appearance, and edge structure) as well as microscopic examination of cellular morphology. Gram staining revealed that the isolates belonged to both Gram-positive and Gram-negative groups and exhibited either rod-shaped or coccoid morphotypes.

For precise taxonomic identification, MALDI-TOF MS analysis was employed. This technique enabled rapid and reliable identification of isolates at the species level through comparison of protein mass spectra with a reference database. The results demonstrated a considerable taxonomic diversity among the isolates, including representatives of the genera *Microbacterium*, *Paenibacillus*, *Pediococcus*, *Bacillus*, *Lactiplantibacillus*, *Weissella*, *Enterococcus*, and *Enterobacte*r.

The taxonomic affiliation of the isolates and their corresponding sources are summarized in Table 1.

Table 1. Taxonomic and Morphological Characteristics of Bacterial and Yeast Isolates

№	Taxonomic Group (MALDI-TOF MS)	Source of Isolation	Colony Morphology	Cell Morphology and Size (µm)
1	*Microbacterium* sp.	Bell pepper	Cream-colored, circular, smooth, slightly glistening, Ø 1–2 mm	Gram (+), short rods, solitary, non-spore-forming. 0.4–0.8×1.0–3.0 µm
2	*Paenibacillus amylolyticus*	Potato	Off-white, irregular margins, flat, Ø 2–5 mm	Gram (+), long rods, arranged in chains, endospores present. 0.6–1.0×2.0–5.0 µm
3	*Pediococcus acidilactici*	Pomegranate interior	White, circular, convex, Ø 0.5–1 mm	Gram (+), cocci, arranged in tetrads or clusters, non-spore-forming. Ø 0.8–1.2 µm
4	*Escherichia coli*	Cucumber, acacia	Off-white, glistening, semi-convex, Ø 1–3 mm	Gram (–), rod-shaped, solitary, non-spore-forming. 0.5–0.8×1.5–3.0 µm
5	*Bacillus megaterium*	Potato	Off-white, large, irregular (rhizoid), serrated margins, Ø 3–6 mm	Gram (+), large rods, solitary or in chains, central spores present. 1.2–1.5×2.0–5.0 µm
6	*Bacillus simplex*	Potato	Cream-colored, irregular undulate margins, flat, Ø 2–4 mm	Gram (+), rod-shaped, forms chains, spores present. 0.7–1.0×2.0–4.0 µm
7	*Candida glabrata* (yeast)	Carrot	Cream-colored, circular, smooth, convex, Ø 2–4 mm	Gram (+), oval or spherical, budding observed, non-spore-forming. Ø 2–5 µm
8	*Weissella confusa*	Maize, cheese	White, circular, smooth, Ø 0.8–1.5 mm	Gram (+), short rods (coccobacilli), arranged in pairs. 0.5–0.8×1.0–2.5 µm
9	*Lactiplantibacillus plantarum*	Cabbage	White, circular, smooth, plano-convex, Ø 1–2 mm	Gram (+), medium-sized rods, chain-forming, non-spore-forming. 0.7–1.0×2.0–5.0 µm
10	*Enterococcus faecalis*	Acacia	Off-white, circular, dull-glistening, Ø 1–2 mm	Gram (+), cocci, pairs or short chains, non-spore-forming. Ø 0.6–1.0 µm
11	*Enterobacter cloacae*	Carrot	Off-white, circular, smooth, mucoid, Ø 2–4 mm	Gram (–), rod-shaped, solitary. 0.3–0.6×0.8–2.0 µm
12	*Kurthia gibsonii*	Carrot	Smooth, cream-yellow, glistening, Ø 1–3 mm	Gram (+), long rods (young culture), short rods/cocci (aged culture), non-spore-forming. 0.8–1.0×2.0–6.0 µm

The diversity of microorganisms isolated from traditional fermented foods highlights the microbiological richness of these products and confirms their potential as valuable sources of functionally important bacterial strains.

3.2. Screening and identification of tannase-producing isolates

The ability of the isolated strains to hydrolyze tannic acid was assessed using the tannin-agar plate method followed by $FeCl_3$ visualization, as described by Kumar et al. (2010). After treatment with ferric chloride, four isolates exhibited distinct brown hydrolysis zones around the colonies, indicating the production of gallic acid and confirming tannase enzyme activity.

The tannase-positive isolates were identified as: *Microbacterium* sp., *Paenibacillus amylolyticus*, *Pediococcus acidilactic*i, and *Enterobacter cloacae*

The detection of tannase activity in *Microbacterium* sp. is consistent with previous reports describing tannase production by members of this genus, particularly *Microbacterium terregens* [14]. Similarly, tannase synthesis by *Enterobacter cloacae* has been documented in studies focusing on the biotransformation of tea polyphenols [15], and the results obtained in the present study are in full agreement with these findings.

In contrast, the identification of tannase activity in *Paenibacillus amylolyticus* represents a novel scientific observation. This species has primarily been reported as a producer of hydrolytic enzymes such as amylases, xylanases, and proteases, involved in the degradation of complex polysaccharides [16]. The present results expand the known enzymatic repertoire of P. amylolyticus and suggest its potential application

in the bioconversion of tannin-rich agricultural residues.

Of particular interest is the detection of tannase activity in *Pediococcus acidilactici*. While tannase production has been extensively studied in *Lactiplantibacillus plantarum* and related lactic acid bacteria [17], reports on tannase activity in *Pediococcus* species remain limited. Therefore, the findings of this study contribute new knowledge to the field and underline the biotechnological relevance of *P. acidilactici* beyond its well-established probiotic properties.

3.3. Evaluation of antioxidant activity

The antioxidant potential of the isolates was evaluated using the DPPH radical scavenging assay. A total of 18 microbial cultures were included in the analysis, comprising newly isolated *Pediococcus acidilactici* strains as well as collection strains of *Lactiplantibacillus plantarum* and *Lactobacillus brevis*.

The results were statistically analyzed in comparison with the positive control (ascorbic acid).

Among the tested strains, *L. plantarum* exhibited the highest DPPH radical scavenging activity (72.8%), followed by *P. acidilactici* (68.4%) and *L. brevis* (65.2%). These values indicate moderate to high antioxidant activity, confirming the ability of lactic acid bacteria to counteract oxidative stress.

The relatively high antioxidant activity observed in *P. acidilactici* is particularly noteworthy, as this strain also demonstrated tannase activity. This dual functionality suggests that *P. acidilactici* may contribute to both tannin degradation and antioxidant defense mechanisms, making it a promising candidate for functional food applications.

The superior antioxidant performance of *L. plantarum* can be attributed to its well-documented genetic capacity for polyphenol metabolism and the synthesis of antioxidant enzymes and bioactive peptides. These findings are consistent with previous studies reporting strong antioxidant properties of *L. plantarum* strains involved in polyphenol-rich food fermentation [15,12].

3.4. Integrated analysis of tannase activity and antioxidant potential

The combined evaluation of tannase production and antioxidant activity highlights the functional relevance of the selected strains. Tannase-positive isolates demonstrated enhanced antioxidant properties, which may be linked to the biotransformation of tannins into lower-molecular-weight phenolic compounds such as gallic acid.

Furthermore, the ability of tannase-producing lactic acid bacteria to reduce tannin content in foods may improve mineral bioavailability, particularly iron, by preventing the formation of insoluble tannin–metal complexes. This characteristic is especially important for the development of functional foods aimed at mitigating micronutrient deficiencies.

CONCLUSION

As a result of this study, **17 pure microbial strains** were isolated from natural sources, and their taxonomic diversity—including the genera *Lactiplantibacillus*, *Pediococcus*, *Microbacterium*, and *Bacillus*—was characterized using **MALDI-TOF MS** analysis. Screening procedures identified high tannase activity in four specific strains. A significant scientific contribution of this work is the first-time verification of tannase activity in strains of *Paenibacillus amylolyticus*, and *Pediococcus acidilactici*.

In antioxidant assays, *L. plantarum* (72.8%) and *P. acidilactici* (68.4%) exhibited the highest radical scavenging capacities. Consequently, these isolated strains are recommended as promising bio-agents for the reduction of anti-nutritional factors in the food industry and for the production of natural antioxidants in the pharmaceutical sector.

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