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Research Article

Assessment of Tannase Producer *Acinetobacter nosocomialis* (MT540255) and *Staphylococcus saprophyticus* (GU197531) Isolated from Ruminant Gut Fluid

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ABSTRACT

Excessive tannin intake in ruminant diets adversely affects feed palatability and nutrient digestibility; however, microbial tannases offer an effective biological approach to tannin degradation. In this study, two previously identified tannase-producing bacterial strains isolated from ruminant gut fluid -- *Acinetobacter nosocomialis* (MT540255) and *Staphylococcus saprophyticus* (GU197531) -- were evaluated for the optimization of physicochemical parameters affecting tannase activity using tannic acid, while assessing their antibiotic resistance profiles and the synergistic antimicrobial activity of crude tannase extracts. Both strains showed morphological and biochemical characteristics consistent with those reported for their respective genera. *A. nosocomialis* (MT540255) exhibited optimal tannase activity at pH 6, 40°C, and 1.6% substrate concentration for 105 min, resulting in a 1.23-fold increase to 42.51 U/mL. In contrast, *S. saprophyticus* (GU197531) reached its peak activity at pH 10, 50°C, and 1.8% substrate concentration for 45 min, yielding a 1.93-fold increase to 48.43 U/mL. Overall, optimization of physicochemical conditions enhanced tannase activity in both strains, with pH emerging as a key contributing factor for *A. nosocomialis* (MT540255), while *S. saprophyticus* (GU197531) showed additional strong responsiveness to pH and substrate concentration. *A. nosocomialis* (MT540255) and *S. saprophyticus* (GU197531) were found to be resistant to six and four of the ten antibiotics tested for antibiotic susceptibility, respectively. Notably, crude tannase from both strains showed significant synergistic effects (p<0.05) when combined with streptomycin. These findings suggest the potential application of tannase-producing bacteria from ruminant gut fluid in improving the nutritional quality of ruminant feed through tannin degradation, while offering synergistic benefits in antimicrobial applications.

Key words: Antibiotic resistance, Acinetobacter strain, characterization, ruminal gut, Staphylococcus strain, tannase

INTRODUCTION

Tannase (tannin acyl hydrolase) is an enzyme widely used across various industries, including food processing, animal feed, cosmetics, pharmaceuticals, chemicals, and leather manufacturing (Al-Mraai *et al*., 2019). This enzyme can be produced by different organisms, yet microorganisms are the main source due to the higher stability and industrial adaptability of microbial enzymes (Patel *et al*., 2022). Traditionally, tannase production has been associated with fungal species, particularly *Aspergillus* and *Penicillium* (Leangnim *et al*., 2023). However, recent research has increasingly focused on bacterial tannases, which offer advantages such as faster growth rates and greater ease of genetic manipulation (Beniwal *et al*., 2014; Dhiman *et al*., 2021). Numerous bacterial genera have been reported for their ability to produce tannase, including *Bacillus, Staphylococcus, Klebsiella, Lactobacillus, Citrobacter, Streptococcus, Pseudomonas, Corynebacterium, Pantoea, Selenomonas, Enterococcus,* and *Serratia* (Mohammed, 2016; Tahmourespour *et al*., 2016; Tripathi *et al*., 2016; Aharwar & Parihar, 2018). Microbial tannases not only have industrial applications but also play an important functional role in animals, linking microbial production to biological significance.

Specifically, tannase catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins (gallotannins & ellagitannins), complex tannins, and condensed tannins (Hassan *et al*., 2020). These tannins are polyhydroxy phenolic compounds present in plants used for food and feed, where they serve as defense mechanisms against herbivores, pathogenic microbes, and insects (Mayer, 2022). Yet tannins are associated with adverse nutritional effects because of their strong ability to form complexes with proteins and other essential nutrients (Hiura *et al*., 2010). Consequently, this interaction disrupts ruminal pH and digestive enzyme activity, leading to reduced nutrient absorption, which in turn affects animal growth (Vandenplas & Devreker, 2019; Hassan *et al*., 2020; Besharati *et al*., 2022). Nevertheless, diverse bacteria residing in the rumen and gastrointestinal tract (GIT) of animals have evolved mechanisms to tolerate and degrade tannins from tannin-rich plants, making them a valuable source for

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isolating tannase-producing strains (Tahmourespour *et al*., 2016; Cipriano-Salazar *et al*., 2018).

The production of tannase from bacterial sources is commonly carried out using submerged fermentation (SF), as it provides high moisture content, shorter doubling time, easier product extraction, and simple fermentation control (Beniwal *et al*., 2014; Jana *et al*., 2015). Furthermore, strain selection and fermentation conditions (pH, substrate concentrations, temperature, incubation time) also influence enzyme productivity, activity, and stability (Fitsum *et al*., 2025). Therefore, physicochemical characterization of tannase has become a major research focus, providing essential information for optimizing its performance and functionality for industrial and biotechnological applications (Chávez-González *et al*., 2012). Although existing literature has largely focused on bacterial tannases, limited information is available on tannases produced by *Acinetobacter nosocomialis* (MT540255) and *Staphylococcus saprophyticus* (GU197531), particularly regarding their enzymatic activity and properties under varying physicochemical conditions.

In addition, tannase catalysis produces gallic acid, a valuable compound with varied pharmacological properties, including antibacterial, antiallergic, antioxidant, antimutagenic, anti-inflammatory, neuroprotective, and anticancer activities (Selvaraj *et al*., 2022). The rising threat of multidrug-resistant bacteria, particularly Gram-negative species, has prompted the exploration of alternative strategies to enhance antimicrobial efficacy using natural agents such as tannase and gallic acid (Hidayathulla *et al*., 2018). Several studies have documented the antimicrobial activity of gallic acid; even so, information regarding the antibiotic resistance profiles of tannase-producing bacteria and the potential synergistic antimicrobial effects of tannase in combination with antibiotics remains limited.

Therefore, this study aims to address several key issues by characterizing the properties of crude tannase produced by two strains of tannase-producing bacteria isolated from ruminant gut fluid using parameters such as pH, substrate concentration, temperature, and incubation time via modified spectrophotometric methods. Furthermore, their antimicrobial resistance and the synergistic effects of crude tannase were analyzed using commercial antibiotics. It is anticipated that the insights acquired from this study will open avenues for an alternative bacterial tannase source to be utilized in various biotechnological applications, especially in ruminant feed production and pharmaceuticals.

MATERIALS AND METHODS

Reculture of tannase-producing bacteria

Acinetobacter nosocomialis (MT540255) and *Staphylococcus saprophyticus* (GU197531) were previously isolated from ruminant gut fluid collected at the Kompleks Abatoir Shah Alam (3.057442° N, 101.519337° E) using minimal salt medium (MSM) agar supplemented with 0.2% tannic acid (Suhaimi *et al*., 2024). The strains were preserved in glycerol stock cultures at -20 ± 2°C. Before each experiment, the bacteria were routinely cultured in nutrient broth with tannic acid (NBTA) and subsequently streaked onto nutrient agar containing tannic acid (NATA) to selectively recover tannase-producing bacteria. The resulting colonies were subcultured onto standard nutrient agar and incubated overnight at 37°C for further analyses.

Morphological and biochemical characterization of isolates

The isolates were characterized based on their colonial morphology using six parameters: colony size, opacity, texture, form, margin, and elevation. In addition, the isolates were subjected to phenotypic characterization through Gram-staining and standard biochemical tests, including indole, catalase, oxidase, triple sugar iron (TSI), and Methyl Red–Voges Proskauer (MR–VP).

Tannase activity and growth curve of bacterial isolates

Tannase production was performed according to the method described by Balakrishnan *et al*. (2021) with minor modifications. The MSM broth used as the fermentation medium consisted of the following constituents (g/L): tannic acid (10) as the carbon source, NH_4Cl (1) as the nitrogen source, KH_2PO_4 (0.5) as the phosphate source, $MgSO_4$ (0.5), K_2HPO_4 (0.5), $CaCl_2$ (0.01) as metal ions, and glucose (0.5). A 100 mL portion of the medium with pH 5.0 ± 0.2 was placed in 500 mL Erlenmeyer flasks and sterilized. Subsequently, 1 mL of the overnight bacterial culture was inoculated into the prepared MSM broth and incubated in an incubator shaker at 37°C and 180 rpm for 5 days. Samples were collected daily, centrifuged at 4000 rpm for 10 min at 4°C, and the resulting supernatant was stored as crude enzyme. Bacterial growth was monitored by measuring the optical density of the cultures at 600 nm until a decline was observed. Data are expressed as mean ± standard deviation of triplicate experiments. Differences in tannase production at different incubation times were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. Differences were considered significant at $p<0.05$.

Tannase activity was evaluated using a spectrophotometric assay based on the method described by Srivastava and Kar (2009) with slight modifications. Tannic acid was employed as a substrate, and gallic acid was used to construct the calibration curve. The reaction mixture consisted of 0.5 mL of crude enzyme and 0.25 mL of 0.01 M tannic acid prepared in 0.05 M citrate buffer (pH 5.0), then incubated at 30°C for 10 min. Subsequently, 0.3 mL of methanolic rhodanine (0.667% w/v rhodanine in methanol) was added and left at room temperature for 5 min. Thereafter, 0.2 mL of 0.5 M potassium hydroxide (KOH) was introduced, followed by the addition of 10 mL of distilled water. The mixture was incubated at 30°C for 10 min, and the resulting purple-pink coloration was measured at 520 nm. The tube for the control was represented by the buffer only, whereas the blank, which was represented solely by the substrate, was used for each sample simultaneously (Ire & Nwanguma, 2020). One unit of tannase activity is defined as the amount of enzyme required to release 1 µmoL of gallic acid per min under the given assay conditions. The enzyme activity was expressed in U/mL.

Optimization of different physicochemical parameters on tannase activity

The maximum tannase production was assessed under different physicochemical conditions. Crude enzyme extracts were incubated with substrate in citrate buffer after adjusting the respective parameters. The evaluated parameters included pH (3.0

to 10.0), substrate concentration (0.2% to 2%), temperature (30°C to 60°C), and incubation time (15 min to 120 min) (Shakir *et al*. 2022). Each parameter was optimized sequentially using a one-factor-at-a-time (OFAT) approach, and the optimum value of each parameter was kept constant while optimizing the next parameter. The residual enzyme activity was determined using the standard enzyme assay procedure. All experiments were carried out in triplicate, and the mean values were reported with the standard error.

Antibiotic susceptibility testing and synergistic crude tannase effects

The antibiotic susceptibility testing (AST) of tannase-producing bacteria was performed using the disc diffusion method on Mueller-Hinton agar plates, in accordance with the guidelines provided by the Clinical Laboratory Standards Institute (CLSI). The antibiotics tested included ampicillin/sulbactam (AMP/SUL 20 µg), ciprofloxacin (CIP 5 µg), cefoxitin (CF 30 µg), chloramphenicol (CHL 30 µg), gentamicin (GEN 10 µg), penicillin-G (PenG 10 iU), tigecycline (TIG 15 µg), streptomycin (STR 10 µg), and vancomycin (VA 30 µg). Bacterial suspensions were prepared in 0.9% saline to a McFarland density of 0.5 and uniformly swabbed in three directions onto agar plates using sterile cotton swabs. Sterile distilled water served as the negative control. Antibiotic discs were placed on the inoculated plates, which were then incubated overnight at 37°C. Afterwards, the diameters of the inhibition zones were recorded. AST was performed in biological triplicate for each sample.

The synergistic antimicrobial activity of four commercial antibiotics -- penicillin-G (PenG 10 iU), gentamicin (GEN 10 µg), streptomycin (STR 10 µg), and tigecycline (TIG 15 µg) – used alone and in combination with crude tannase was evaluated against two Gram-positive strains (Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*) and one Gram-negative strain (*Klebsiella pneumoniae*) using the same disc diffusion method. A volume of 20 µl crude tannase extracted from each isolate was loaded onto sterile discs and placed on agar plates that were inoculated with the respective test strains. Citrate buffer served as the negative control. The plates were incubated overnight at 37°C, and the zones of inhibition were measured. All experiments were performed in triplicate, and the mean values are presented. The increase in fold area was assessed by calculating the mean surface area of the inhibition zone for each antibiotic alone and in combination with the crude tannase. The fold increase was calculated using the formula $(B^2 - A^2)/A^2$, where A and B represent the inhibition zone diameters for antibiotics alone and antibiotics combined with crude tannase, respectively.

RESULTS

Morphological and biochemical characterization of isolates

The morphological and biochemical characteristics of two tannase-producing bacterial strains isolated from ruminant gut fluid – *Acinetobacter nosocomialis* (MT540255) and *Staphylococcus saprophyticus* (GU197531) – are presented in Table 1. *A. nosocomialis* (MT540255) exhibited large, irregular translucent colonies with a uniformly raised elevation and appeared as Gram-negative coccibacilli in short chains, consistent with characteristics of the *Acinetobacter* genus. Conversely, *S. saprophyticus* (GU197531) formed small, circular, opaque colonies with convex and Gram-positive cocci in clusters, which aligns with the typical *Staphylococcus* genus.

Both isolates showed similar biochemical characteristics, testing negative for indole and oxidase, while showing positive for the catalase test. However, the two isolates exhibited distinct fermentation profiles. *A. nosocomialis* (MT540255) showed a red slant and yellow butt in the TSI test (K/A), indicating fermentation of glucose only, and tested negative for both MR-VP tests. In contrast, *S. saprophyticus* (GU197531) exhibited a yellow slant and yellow butt in the TSI test (A/A), demonstrating the ability to ferment glucose, lactose, and sucrose, while testing positive for both MR-VP tests.

Table 1. Morphological and biochemical characterization of two tannase-producing bacteria isolated from ruminant gut fluid

Morphological and biochemical characterization	*A. nosocomialis* (MT540255)	*S. saprophyticus* (GU197531)
Size	Large	Small
Opaqueness	Translucent	Opaque
Form	Irregular	Circular
Margin	Entire	Entire
Elevation	Raised	Convex
Gram-staining	Negative	Positive
Indole	Negative	Negative
Oxidase	Negative	Negative
Catalase	Positive	Positive
Triple Sugar Iron (TSI)	K/A[#]	A/A[#]
Methyl Red (MR)	Negative	Positive
Voges-Proskauer (VP)	Negative	Positive

[#]K/A = alkaline slant/acid butt; A/A = acid slant/acid butt

Tannase production and growth curve of bacterial isolates

Bacterial growth and tannase production showed a positive correlation with increasing incubation time, as illustrated in Figure 1. The exponential growth phase of *A. nosocomialis* (MT540255) isolates occurred from day 0 to day 2, whereas for *S. saprophyticus* (GU197531) isolates, it extended from day 0 to day 3. This phase aligned with the peak tannase activity observed for each isolate: 34.62 U/mL [*A. nosocomialis* (MT540255)] and 25.08 U/mL [*S. saprophyticus* (GU197531)]. Subsequently, both bacterial isolates entered the stationary phase on day 4, marked by a decline in the growth curve and a decrease in enzyme activity.

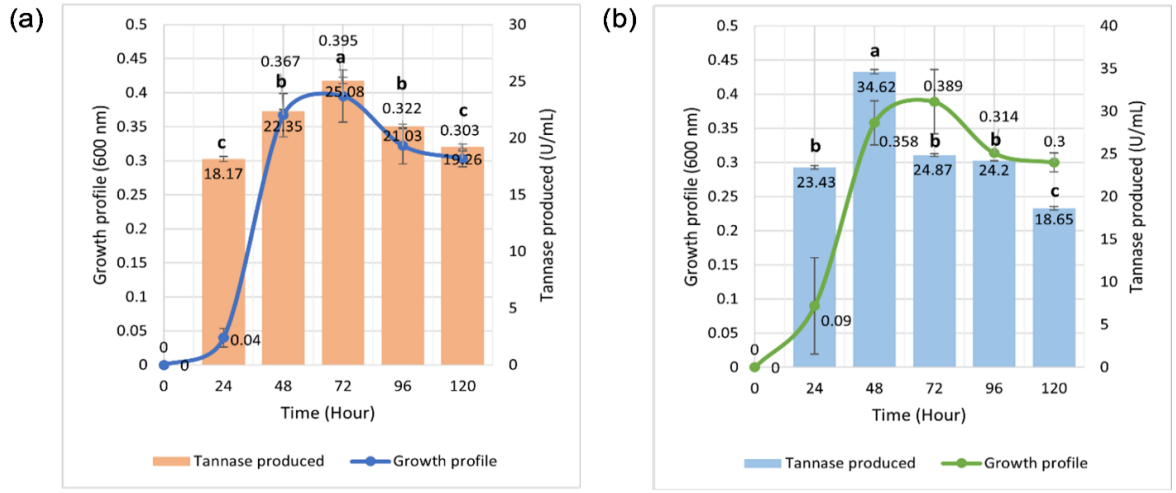

Fig. 1. Tannase production and bacterial growth during five days of fermentation under initial conditions. Fermentation was carried out in MSM broth adjusted to pH 5 and supplemented with 0.6% (w/v) tannic acid as the substrate at 37°C. Tannase activity (U/mL) and bacterial growth, measured as optical density at 600 nm, were recorded daily for five days. Bar charts represent tannase activity, while line plots indicate bacterial growth**. (a)** *Acinetobacter nosocomialis* (MT540255), **(b)** *Staphylococcus saprophyticus* (GU197531). Values differ significantly between groups at $p<0.05$.

Optimization of different physicochemical parameters on tannase activity

The activity of crude tannase was assessed under different physicochemical conditions to determine the optimal parameters for maximum enzyme performance. The enzyme exhibited stability at pH values above pH 5 (Figure 2). Optimal tannase production by *A. nosocomialis* (MT540255) was observed at pH 6, with an activity of 34.62 U/mL, showing increased activity under slightly acidic conditions and a decline in more alkaline environments. In contrast, *S. saprophyticus* (GU197531) demonstrated a gradual increase in tannase activity with increasing pH, reaching a peak of 25.08 U/mL at pH 10 before decreasing. These findings suggest that enzyme production is favored under slightly acidic to alkaline conditions. Further analysis of substrate tolerance revealed that *A. nosocomialis* (MT540255) produced 41.36 U/mL of tannase at 1.6% tannic acid, while *S. saprophyticus* (GU197531) showed a slightly higher production of 41.48 U/mL at 1.8% substrate concentration (Figure 3). Temperature also significantly influenced enzyme production (Figure 4). *A. nosocomialis* (MT540255) reached its highest activity (42.38 U/mL) at 40°C, while *S. saprophyticus* (GU197531) achieved maximum production (42.08 U/mL) at 50°C, with activity declining beyond these temperatures. Incubation time was another key factor affecting enzyme yield (Figure 5). Tannase activity was first detected at 15 min and continued to rise over time. Remarkably, *A. nosocomialis* (MT540255) achieved peak activity of 42.51 U/mL at 105 min, whereas *S. Saprophyticus* (GU197531) reached a higher peak of 48.43 U/mL at 45 min. *S. Saprophyticus* (GU197531) exhibited a higher fold increase (1.93) compared to *A. nosocomialis* (MT540255) (1.23) under optimized conditions.

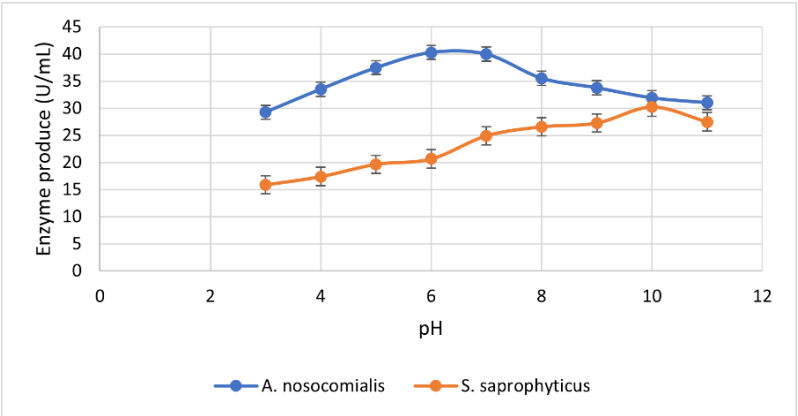

Fig. 2. Tannase activity (U/mL) of tannin-degrading bacteria across varying pH levels. (Control:green line = No bacteria; blue line = *A. nosocomialis* (MT540255); orange line = *S. saprophyticus* (GU197531)). Measurements represent mean values from triplicate experiments.

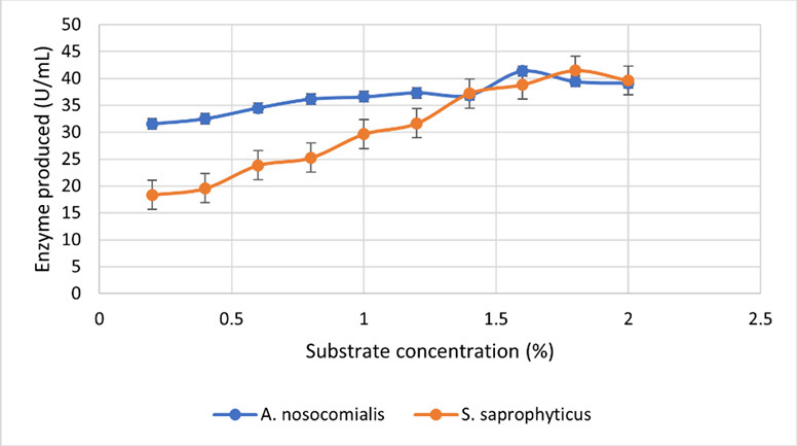

Fig. 3. Tannase activity (U/mL) of tannin-degrading bacteria across varying substrate concentrations. (Control:green line = No bacteria; blue line = *A. nosocomialis* (MT540255); orange line = *S. saprophyticus* (GU197531)). Measurements represent mean values from triplicate experiments.

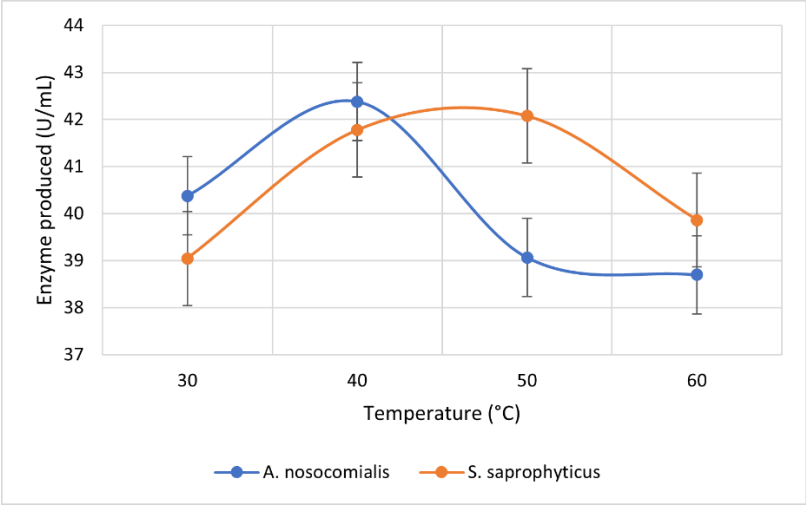

Fig. 4. Tannase activity (U/mL) of tannin-degrading bacteria across varying temperatures. (Control:green line = No bacteria; blue line = *A. nosocomialis* (MT540255); orange line = *S. saprophyticus* (GU197531)).Measurements represent mean values from triplicate experiments.

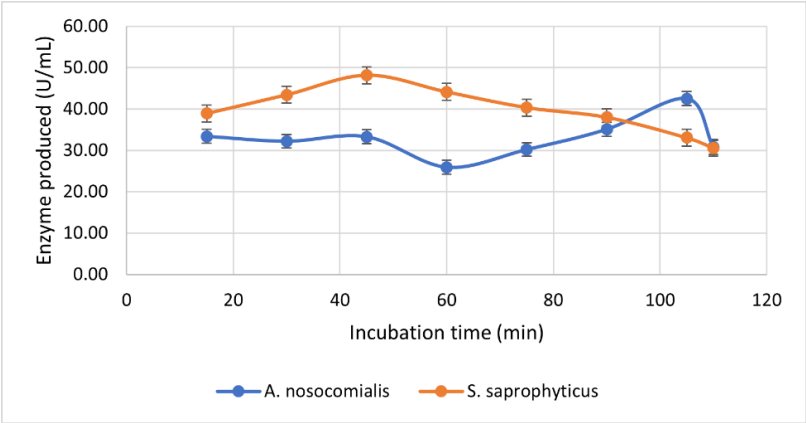

Fig. 5. Tannase activity (U/mL) of tannin-degrading bacteria across varying incubation time. (Control:green line = No bacteria; blue line = *A. nosocomialis* (MT540255); orange line = *S. saprophyticus* (GU197531)).Measurements represent mean values from triplicate experiments.

Table 2. Optimization of tannase activity by *Acinetobacter nosocomialis* (MT540255) and *Staphylococcus saprophyticus* (GU197531) in different physicochemical conditions

Parameter		*A. nosocomialis* (MT540255)	*S. saprophyticus* (GU197531)
Before optimization	Tannase activity (pH 5, 0.6% tannin, 37°C)	34.62 U/mL	25.08 U/mL
pH	Optimal	6	10
	Tannase activity	40.28 U/mL	30.22 U/mL
Substrate concentration	Optimal	1.6%	1.8%
	Tannase activity	41.36 U/mL	41.48 U/mL
Temperature	Optimal	40°C	50°C
	Tannase activity	42.38 U/mL	42.08 U/mL
Incubation time	Optimal	105 min	45 min
	Tannase activity	42.51 U/mL	48.43 U/mL
	Fold increment	1.23	1.93

Fold increment represents the increase in tannase activity relative to the initial unoptimized conditions.

Antibiotic susceptibility testing and synergistic crude tannase effects

The susceptibility patterns of each bacterium to the standard commercial antibiotics were presented in Table 3. *A. nosocomialis* (MT540255) exhibited resistance to six (6) antibiotics: penicillin G (PenG), chloramphenicol (CHL), ciprofloxacin (CIP), cefoxitin (CF), tigecycline (TIG), and vancomycin (VA). In contrast, *S. saprophyticus* (GU197531) demonstrated resistance to four (4) antibiotics only: ampicillin/sulbactam (AMP/SUL), penicillin G (PenG), cefoxitin (CF), and tigecycline (TIG).

Table 3. Means of inhibition zones (mm) of each isolated tannase-producing bacteria against 10 types of commercial antibiotics

Commercial antibiotics	Means of inhibition zones of standard antibiotic discs against tested bacterial strains (mm)	
	Acinetobacter nosocomialis strain AE6 (MT 540255)	*Staphylococcus saprophyticus* strain YSY1-3 (GU 197531)
Ampicillin/Sulbactam (20 µg)	S (19.3 ± 0)**	R (0 ± 0)**
Penicillin G (10 iU)	R (11 ± 0.17)*	R (0 ± 0)*
Chloramphenicol (30 mcg)	R (10 ± 0)*	S (29 ± 0)*
Ciprofloxacin (5 µg)	R (11.7 ± 1.36)*	S (29.7 ± 0.06)
Cefoxitin (30 mcg)	R (0± 0)*	R (0 ± 0)*
Tigecycline (15 µg)	R (9± 0.10)*	R (10 ± 0)*
Vancomycin (30 µg)	R (0 ± 0)*	S (18± 0)
Gentamicin (10 µg)	I (15.3 ± 0.12)*	S (30.7 ± 0.12)*
Streptomycin (10 µg)	S (19 ± 0.17)*	S (26.7 ± 0.06)*

R = Resistant; I = Intermediate; S = Susceptible

* - Significant (p<0.05); ** - Insignificant (p>0.05)

± indicates standard deviation.

The inhibition zone was analyzed according to the CLSI Standard for each bacterium.

The antibacterial effects of crude tannase from two isolates, both individually and in combination with antibiotics, were evaluated against selected bacterial strains, revealing distinct inhibitory patterns (Table 3). Crude tannase extracted from each isolate exhibited significant antibacterial activity against all tested bacteria, with inhibition zones ranging from 8 mm to 13 mm, except against MRSA, which showed no inhibition. Notably, the combination of crude tannase with antibiotics enhanced the inhibition against several bacterial strains compared to antibiotics alone, indicating a synergistic interaction. Among the combinations tested, crude tannase from *A. nosocomialis* (MT540255) showed the highest synergistic activity with streptomycin against *S. epidermidis*, producing an inhibition zone of 10 ± 14.14 mm and a fold area increase of 1.78, compared to no inhibition with streptomycin alone. Similarly, it also demonstrated notable activity against MRSA when combined with streptomycin, with an inhibition zone of 20 ± 2.83 mm and a fold area of 0.66. Meanwhile, the combination of crude tannase from *S. saprophyticus* (GU197531) with meropenem showed the highest fold area at 3.00 against *S. epidermidis,* with the inhibition zone increasing from 0 to 12 ± 1.4 mm. Additionally, it exhibited increased activity when combined with penicillin G and tigecycline against *S. epidermidis*, with fold area increases of 0.84 and 1.25, respectively.

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Table 4. Synergistic antibacterial activity of crude tannase in combination with four antibiotics against MRSA, *K. pneumoniae,* and *S. epidermidis*

Extract from isolate	Test strains	Mean inhibition zone (mm) of antibiotic discs in combination with the crude tannase against clinical bacterial strains[1]											
		CT	P	P + CT	Fold area	GN	GN + CT	Fold area	S	S + CT	Fold area	TGC	TGC + CT	Fold area
MT 540255	MRSA	0	16 ± 9.0	16 ± 0.7	0	18 ± 6.0	18 ± 6.0	0	15 ± 0.71	20 ± 2.83	0.66	20 ± 0	21 ± 1.41	0.10
	K. pneumoniae	10	18 ± 2.0	18 ± 2.8	0.06	14 ± 1.0	15 ± 0	0.07	16 ± 1.41	16 ± 1.41	0	22 ± 2.12	22 ± 2.83	0.05
	S. epidermidis	13	9 ± 12	10 ± 13.4	0.3	14 ± 20	16 ± 22	0.2	0	10 ± 14.14	1.78	13 ± 1.84	14 ± 1.91	0.08
GU 197531	MRSA	6	0	0	0	18 ± 2.3	18 ± 2.3	0.0	16 ± 1.0	16 ± 1.0	0	24 ± 1.5	26 ± 1.2	0.17
	K. pneumoniae	8	0	0	0	14 ± 3.5	15 ± 3.1	0.15	16 ± 0.6	17 ± 0.6	0.13	24 ± 1.5	25 ± 1.2	0.09
	S. epidermidis	9	14 ± 0.7	19 ± 0	0.84	22 ± 1.4	24 ± 1.4	0.19	0	12 ± 1.4	3.00	16 ± 0.7	24 ± 0.7	1.25

CT – Crude Tannase; P – Penicillin G (10 µg); GN – Gentamicin (10 µg); S – Streptomycin (30 µg); TGC – Tigecycline (25 µg).

[1]Mean surface area of the inhibition zone was calculated for each from the mean diameter. ± indicates standard deviation.

Increase in fold area was calculated as = $(y^2 - x^2)/x^2$, where 'x' and 'y' are the inhibition zones for antibiotic and antibiotic + tannase, respectively.

*In the absence of bacterial growth inhibition zones, the disk's diameter (6 mm) was used to calculate the fold increases.

Red – Resistant; Green – Intermediate; Blue – Susceptible

DISCUSSION

The ruminant gastrointestinal tract represents a complex ecosystem that harbors diverse microbial communities essential for nutrient metabolism and detoxification of plant secondary compounds (Fu *et al*., 2025). Ruminants are naturally exposed to tannins, as these compounds are commonly present in plant-based diets (Kadigi *et al*., 2024). Consequently, rumen microbes have evolved mechanisms to tolerate or degrade tannins by producing tannase, an enzyme that enables them to persist in tannin-rich environments (Tahmourespour *et al*., 2016). Several bacterial strains capable of degrading tannins have been isolated from various sources, including goat feces (Tahmourespour *et al*., 2016), rumen (Gheibipour *et al*., 2022), and the gut of *Gryllotalpa krishnani* insects (Govindarajan *et al*., 2016). In this study, two bacterial strains -- *Acinetobacter nosocomialis* (MT540255) and *Staphylococcus saprophyticus* (GU197531) – previously isolated from ruminant gut fluid, demonstrated tannin-degrading capability in preliminary screening through the formation of clear hydrolysis zones on MSM agar plates supplemented with tannic acid (Suhaimi *et al*., 2024).

The MSM tannic acid broth medium has been widely used for tannase production and supports optimal growth of several tannase-producing bacteria, including *Cryptococcus sp*. NRC10, *Bacillus subtilis* KMS2-2, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Streptomyces olivochromogenes* BH49, after 24 hr of incubation at 37°C (Tork, 2020; Govindarajan *et al*., 2021; Teron *et al*., 2021). This aligns with our study, where both *A. nosocomialis* (MT540255) and *S. saprophyticus* (GU197531) fermented efficiently under initial conditions of pH 5, 37°C, and 0.6% tannic acid, with maximum growth observed at 48 hr and 72 hr, respectively, which corresponded with increased tannase production. Although other studies have reported high tannase production in *Acinetobacter baumannii* at 26.46 mg/mL after 72 hr of incubation (Abdulshaheed *et al*., 2023) and notable tannase activity in *Staphylococcus aureus* at 3.81 U/min after 24 hr of fermentation (Isah *et al*., 2017), enzyme production is generally associated with the exponential growth phase and declines when nutrient limitations occur (Jebur, 2020). Importantly, direct comparison of tannase yields between our isolates and previously reported strains is limited, as *A. nosocomialis* (MT540255) and *S. saprophyticus* (GU197531) have only recently been identified as tannase-producing bacteria in ruminant gut environments.

Most bacterial tannases are inactive under highly acidic conditions, likely due to enzyme denaturation, and generally exhibit optimal activity within a pH range of 4.0 to 8.0, whereas fungal tannases possess superior activity at pH 6.0 (Leangnim *et al*., 2023). This trend aligns with our findings for *A. nosocomialis* (MT540255), which reached peak tannase activity at pH 6.0—slightly higher than the optimum pH of 5.5 reported for *Acinetobacter baumannii* by Abdulshaheed *et al*. (2023). Yet the potential of *Acinetobacter* species as tannase producers was only recently identified by Mohammadabadi *et al*. (2021), and their study did not provide comprehensive details on enzymatic activity. Similar acidic pH optima (pH 5-6) have also been reported for tannase produced by *Bacillus* sp, *Klebsiella* sp, and *Lactobacillus* sp. (Kumar *et al*., 2019; Mohapatra *et al*., 2020; Dhiman *et al*., 2021; Shakir *et al*., 2022). Meanwhile, *S. saprophyticus* (GU197531) demonstrated optimal tannase activity at pH 10, which deviates markedly from the typical pH range reported for most bacterial strains. This observation contrasts with *Staphylococcus lugdunensis,* which showed best activity at pH 7 (Chaitanyakumar and Anbalagan, 2016). Similarly, Shakir *et al*. (2022) reported a significantly high tannase activity (218.38 U/mL) at pH 8 for *Bacillus subtilis*, isolated from the gut of *Catla catla* fish. Nevertheless, the alkaline optimum observed in *S. saprophyticus* (GU197531) represents a novel finding among ruminant-associated tannase producers, suggesting that this strain may possess unique structural or regulatory features that enable effective tannin degradation in more alkaline environments. The variations in enzyme production under different pH conditions are likely attributed to the influence of pH on ionic and hydrogen bonding, which are essential for maintaining the enzyme's structural integrity and, consequently, its functionality (Eed, 2013).

Jebur (2020) reported an optimal tannic acid concentration of 2% for crude tannase activity in a free-cell form, yet most bacterial tannases reach maximal activity at lower levels, typically between 0.5% and 1.0% (Kumar *et al*., 2019; Mohapatra *et al*., 2020; Shakir *et al*., 2022). Higher substrate optima are uncommon, with only isolated reports such as *Bacillus haynesii* at 2.5% (Dhiman *et al*., 2021), suggesting that high-tannin tolerance is not a general feature of microbial tannases. In the present study, both isolates showed maximal tolerance at 1.5% tannic acid, beyond which enzyme activity declined, indicating that *Acinetobacter* sp. (MT540255) and *Staphylococcus* sp. (GU197531) may be less adapted to high tannic acid concentrations than some previously reported strains. Although limited data are available for *Acinetobacter*, Abdulshaheed *et al*. (2023) reported tannase production by *A. baumannii* at a comparatively lower tannic acid concentration of 0.5%. Likewise, *Staphylococcus aureus* achieved peak enzyme activity of 3.81 U/min following a 24-hr incubation with 0.2% of tannic acid (Isah *et al*., 2017). The differences in tannase production could be due to substrate specificity. This study used only crude tannase from the isolates, which contains other proteins and components besides the desired enzyme. The presence of these additional enzymes may affect the substrate–enzyme interaction, making it less specific and resulting in lower enzyme activity (Patil *et al*., 2011).

Moreover, temperature is a crucial factor affecting enzyme production, as it influences both enzyme synthesis and cellular viability. Most bacterial tannases reported in the literature exhibit optimal activity within the mesophilic range of 30-40°C, reflecting the physiological growth conditions of many tannase-producing bacteria (Kumar *et al*., 2019; Mohapatra *et al*., 2020; Dhiman *et al*., 2021; Shakir *et al*., 2022). In the present study, *Acinetobacter nosocomialis* (MT540255) demonstrated maximal crude tannase activity at 40°C, aligning well with previously reported optima for bacterial tannases, including those produced by *Bacillus* sp. (Unban *et al*., 2020; Dhiman *et al*., 2021), *Klebsiella pneumoniae (*Kumar *et al*., 2019*)*, *Lactobacillus* sp., and *Acinetobacter baumannii* (Abdulshaheed *et al*., 2023). In contrast, *Staphylococcus saprophyticus* (GU197531) exhibited peak tannase activity at 50°C, indicating enhanced thermal tolerance compared to most reported bacterial tannases. This observation agrees with previous findings showing that tannase from *S. lugdunensis* retained 90% activity at 50°C (Chaitanyakumar & Anbalagan, 2016). Such elevated temperature optima are commonly associated with increased structural stability of the enzyme, allowing sustained catalytic activity under partially denaturing conditions (Farhan *et al*., 2025). Temperature will give effect on covalent and hydrogen bonding and can cause changes in the enzyme's flexibility. The structure of a protein is determined by its chemical bonds, and its function is closely tied to its shape. Therefore, enzyme activity may decrease, or the catalytic process may be disrupted (Eed, 2013). Beyond this, at higher temperatures, tannase activity declined due to the excess potential energy disrupting weak bonds in the enzyme's three-dimensional (3D) structure, leading to denaturation and inactivation of both the enzymes and the substrates (Cavalcanti *et al*., 2018).

Incubation time is crucial for facilitating optimal interaction between tannic acid and tannase, ensuring adequate catalysis for the conversion of tannic acid into gallic acid. Shakir *et al*. (2022) reported that *B. subtilis* achieved maximum tannase activity after 30 min (233.34 U/mL), while *Raoultella ornithinolytica* reached peak activity at 60 min (194.07 U/mL). A significant variation in tannase production during the incubation period was observed among bacterial strains, indicating that enzyme production primarily occurs during the active growth phase. As a result, enzyme activity gradually declines during the stationary phase of tannase production, eventually leading to enzyme degradation in the final stage, likely due to denaturation after prolonged incubation (Govindarajan *et al*., 2019; Jebur, 2020).

In this antibiotic resistance profiling, the *A. nosocomialis* (MT540255) exhibited significant resistance to more tested antibiotics (6) compared to *S. saprophyticus* (GU197531) (4), indicating a higher level of antimicrobial resistance. This finding is consistent with the well-established fact that *Acinetobacter* species are often multidrug-resistant pathogens. Basatian-Tashkan *et al*. (2020) reported that the majority of *A. baumannii* isolates were resistant to ampicillin-sulbactam (65%), ciprofloxacin (61.6%), and gentamicin (48.4%). However, *A. nosocomialis (MT540255)* was found to be susceptible to ampicillin-sulbactam in our findings. According to Manchanda *et al*. (2010), ciprofloxacin resistance in *Acinetobacter* spp. Ranges from 10% to 51%, which supports the variability observed in resistance levels. Moreover, ciprofloxacin and gentamicin are among the most frequently used antibiotics against *Acinetobacter* spp., as reported by Milligan *et al*. (2023). Thus, the resistance and intermediate susceptibility observed to these antibiotics in this study, respectively, could be a consequence of selective pressure resulting from their extensive clinical use. Meanwhile, the susceptibility pattern of *S. saprophyticus* (GU197531) to ciprofloxacin and vancomycin observed in this study aligns with findings from previous studies. Torimiro and Torimiro (2012) reported that *S. aureus* strains were susceptible to both antibiotics. Similarly, vancomycin has been identified as the most effective antibiotic against *S. epidermidis*, with an efficacy rate of 81.2% (Bashir *et al*., 2007).

To the best of current knowledge, this is the first study to characterize the antibacterial activity of tannase derived from bacterial species. The observed synergistic effect of fungal tannase when combined with antibiotics is consistent with previous findings reported by Hidayathulla *et al*. (2018) and Kang *et al*. (2018), which demonstrated enhanced antibacterial activity against *Streptococcus agalactiae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Klebsiella pneumonia*, as evidenced by reduced minimum inhibitory concentrations (MIC) and increased inhibition zone diameters. Muslim *et al*. (2017) also found that fungal tannase enhanced the antibacterial effect of ceftazidime against *Pseudomonas aeruginosa* and *S. aureu*s, showing a synergistic effect even at low ceftazidime concentrations. In addition, Abdulshaheed *et al*. (2023) were the first study to report on purified tannase extracted from *A. baummanii, which* exhibited antibacterial activity against *E. coli, E. cloacae*, *K. pneumoniae*, *S. marcescens*, *P. aeruginosa,* and *S. aureus*, with inhibition zones ranging from 20 to 30 mm.

Although previous studies have primarily focused on fungal tannase, similar synergistic effects were also observed in the present study using bacterial-derived tannase, indicating that tannase from different biological sources may exhibit comparable antibacterial enhancement when combined with antibiotics. Nevertheless, the mechanisms underlying the synergistic effect of the interaction between tannase and antibiotics remain unclear. Hidayathulla *et al*. (2018) proposed that it may involve inhibition of bacterial adhesion or interference with quorum sensing (QS). Their findings demonstrated that combining tannase with antibiotics improves antibacterial efficacy and reduces biofilm formation across several bacterial strains, suggesting the potential of tannase as a novel antibacterial agent. The combination of tannase with antibiotics, such as streptomycin and ceftazidime, further supports its strong antibacterial and anti-biofilm capabilities. This finding highlights the antibacterial properties of tannase and its potential as a novel antibacterial agent.

Dusane *et al*. (2015) have suggested that the hydrolysis of tannic acid involves the cleavage of ester bonds, leading to the formation of gallic acid (GA) and a consequent loss of the antimicrobial activity of tannic acid. Therefore, the observed synergistic antibacterial effects of tannase in this study may be partly attributed to GA, as the extracted tannase was not purified. Previous studies have demonstrated that GA exhibits antibacterial activity against strains such as *E. coli*, *P. aeruginosa*, *S. aureus*, and *Listeria monocytogenes*, causing membrane pores and leading to irreversible changes by altering membrane permeability, hydrophobicity, and physicochemical properties (Borges *et al*., 2013). Similarly, Lu *et al*. (2021) reported that GA exerts strong antibacterial effects against both Methicillin-Sensitive *S. aureus* (MSSA) and Methicillin-resistant *S. aureus* (MRSA) by disrupting membrane adhesions and increasing membrane permeability. In addition to its direct antibacterial activity, GA has been shown to enhance the efficacy of several antibiotics, including ciprofloxacin, erythromycin, norfloxacin, oxacillin, ampicillin, gentamicin, and penicillin (Rajamanickam *et al*., 2019). Given the increasing prevalence of antibiotic resistance, such combination strategies are considered essential for improving therapeutic efficacy and limiting the spread of resistant strains (Buchmann *et al*., 2022). Supporting this, Hossain *et al*. (2020) reported synergistic antibacterial effects of GA in combination with thiamphenicol against *E. coli*. Thus, future studies could further investigate the mechanisms of action and explore the clinical applications of this synergistic approach in antibacterial therapy.

Overall, *S. saprophyticus* (GU197531) demonstrated the highest crude tannase activity at 48.43 U/mL under optimized conditions, indicating robust enzyme production potential. The combination of high tannase activity, alkaline tolerance, and moderate thermostability positions this strain as a promising candidate for biotechnological applications. Notably, its ability to function under rumen-like conditions suggests potential use as a feed additive or direct-fed microbial (DFM) to mitigate tannin toxicity and improve rumen fermentation (Gheibipour *et al*., 2022). In addition, further studies on the tannase produced by this strain, including enzyme purification, detailed stability profiling, antimicrobial activity assessment, and in vivo evaluation for animal feed, are required to validate its practical applicability.

CONCLUSION

This study indicated that tannase activity increased after optimization of physicochemical conditions and demonstrated the synergistic antimicrobial effect of crude tannase produced by two strains isolated from ruminant gut fluid. *Acinetobacter nosocomialis* (MT540255) and *Staphylococcus saprophyticus* (GU197531) exhibited characteristic traits typical of their respective genera. Notably, *S. saprophyticus* (GU197531) was identified as an efficient tannase producer, showing high enzyme

activity (48.43 U/mL) with a 1.93-fold increase after optimization. Moreover, this strain presented a lower level of antibiotic resistance compared to *A. nosocomialis* (MT540255), as resistance was detected in only four of the ten antibiotics tested. In addition, both strains showed significant synergistic effects when combined with streptomycin. Future studies should explore the incorporation of these strains into feed formulations while prioritizing the purification and characterization of tannase to elucidate its antimicrobial properties and pharmacological potential. These endeavors would strengthen the evidence for using ruminant gut bacteria as alternative tannase sources to reduce tannin content in animal feed, thereby supporting the development of sustainable agriculture and animal nutrition.

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ETHICAL STATEMENT
Not applicable.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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