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Optimization of Cultural Conditions for Production of Tannase from a Newly Isolated Strain *Enterobacter hormaechei* PA2

PRIYA, MANJIT K. SELWAL AND KRISHAN KUMAR SELWAL*

Department of Biotechnology, Deenbandhu Chhotu Ram University of Science and Technology, Murthal, Sonipat-131 039 (Haryana), India
**(e-mail: krishan.kselwal@gmail.com; Mobile: 94660 69167)*

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ABSTRACT

Tannase is an inducible enzyme that catalyzes hydrolysis of tannic acid to release glucose, gallic acid, ellagic acid and propyl gallate. In this study, a tannase-producing bacterial strain from termite gut was isolated using 0.3% tannic acid containing medium. The isolate exhibited tannase activity by displaying a halo around the colony. The bacterial culture was identified using biochemical tests, 16S rRNA gene amplification and its subsequent sequencing. Among different substrates tested, the bacterial culture produced maximum enzymatic unit (7.467 U/ml) with betel nuts (*Areca catechu*) as substrate incubated at 37°C temperature. Various physico-chemical parameters were evaluated and it was found that 2.5% inoculum level produced highest enzyme when incubated at 37°C for 48 h. The enzyme activity was enhanced by the addition of ammonium nitrate as a nitrogen source and maltose as a carbon source in the fermentation medium. Moreover, sodium nitrate and triton-X were proved to increase enzyme production as metal ions and additives source, respectively. The results highlighted the presence of tannin-degrading bacterial species within the termite's digestive tract that might prevent it from the harmful effects of tannins within the gastrointestinal environment.

Key words: Tannase, termite, *Areca catechu,* 16S rRNA amplification, solid state fermentation

INTRODUCTION

Tannins are wide spread in nature, such as in leaves, fruits, seeds, wood, bark and considered as the second most abundant group of phenolics after lignin (Aharwar and Parihar, 2018). Tannins can be categorized into two main groups: Hydrolysable tannins that consist of a carbohydrate core, with hydroxyl groups esterified with phenolic acids, and condensed tannins that are high in molecular weight un-branched polymers composed of flavonoids. Tannase (Tannin acyl Hydrolase) is a membrane bound, extracellular enzyme that catalyzes the hydrolysis of ester bonds between galloyl groups and hydroxyl groups of tannins. The presence of tannase-producing microbial species has been well documented from various sources such as, soil (Patil *et al.,* 2020), animal gut like mole cricket (Govindarajan *et al.*, 2016), freshwater fishes (Talukdar *et al.*, 2016), volvano rabbit (Montes-Carreto *et al.*, 2021) and migratory goats (Sharma *et al.*, 2017).

The termite gut is also having a diverse ecosystem and is a home of more than 200 bacterial species, mainly belonging to the phylum firmicutes, such as Spirochaetes, Bacteroidetes, Elusimicrobia, Lactobacillus and Bacillus (Peterson and Scharf, 2016; Ali *et al.*, 2019). Termites are saprophytic in nature and feed, on tannin-rich and decaying plant matter. Moreover, the micro-flora particularly bacteria, could degrade tannins in their natural environments; hence, thrive in the tannins soup. For example, bacterial species such as *Klebsiella variicola*, *Bacillus subtilis*, and *Brevibacillus agri* from the gastrointestinal region of fish have been established (Talukdar *et al.*, 2016). Similarly, Govindarajan *et al.* (2016) confirmed *Enterobacter cloacae* from the gut of Indian mole cricket produced tannase enzyme that gives ecological advantages to circumvent the negative impact of plant tannin.

The present study was aimed at assessing the existence of tannase-producing bacterial isolate in the gastrointestinal tracts of termite. The study isolated autochthonous tannin degrading bacteria from termite gut and the results showed that the saprophytes harbored tannase producing bacteria.

MATERIALS AND METHODS

Termites were collected by manual excavation of their nests and stored in sterilized containers. The samples were placed in an ice box and promptly transported to the laboratory for subsequent work. The termites were washed thrice with sterilized distilled water to eliminate any soil contaminants, followed by a rinse with 70% ethanol. The termites were dissected in a laminar air flow and lower abdomen section was then immersed in the saline solution. The contents were vigorously homogenized to mix well before serial dilution was made. Appropriate dilution was then plated on nutrient agar medium supplemented with tannic acid (0.3%). The plates were then incubated at 37°C for 24-48 h. The isolates obtained were screened for tannin hydrolysis zone around the colonies. The positive isolates were selected and sub-cultured in TA-containing nutrient medium.

Arjun bark (*Terminalia arjuna*), betel nuts (*Areca catechu*), gulmohar flowers (*Delonix regia*), keekar leaves (*Acacia nilotica*), jamun leaves (*Syzygium cumini*) and tamarind seeds (*Tamarindus indica*) were collected and dried at 70°C. The raw material then converted into powdered form using a grinder mixer and their tannin content was estimated following protein precipitation method. For this, the dried leaves were chopped, finely ground, immersed in 70% methanol and left overnight at 4°C. Then, 1 ml of the resulting extract was mixed with 3 ml Bovine Serum Albumin (BSA) solution and incubated for 15 min at room temperature. The mixture was centrifuged (5000 x g, 10 min) and the resulting precipitate was dissolved in 3 ml SDS-triethanolamine solution. Absorbance was measured at 530 nm after adding 1 ml $FeCl_3$ reagent.

The tannase determination was performed as per colorimetric method. An overnight-grown culture was centrifuged at 7000 x g for 5 min and the supernatant was used for the enzymatic assay. The assay involved mixing of 0.3 ml TA substrate (0.5% w/v in 0.2 M acetate buffer, pH 5.5) with 0.1 ml of the supernatant obtained above and incubated at 60°C for 1 h. Then, 3 ml bovine serum albumin (1 mg/ml) was added to the reaction mixture to precipitate residual tannic acid. The mixture was then centrifuged at 7000 x g for 5 min to separate the precipitate. The residual TA was dissolved in 3 ml of SDS-triethanolamine solution (1% w/v SDS in 5% v/v tri-ethanolamine solution) and 1 ml of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01N HCl) was added to the mixture and allowed to incubate for 15 min at room temperature. A control was prepared simultaneously containing denatured enzyme with TA. The absorbance was determined at 530 nm and enzymatic unit was calculated using a standard calibration curve. One unit of enzyme was defined as the quantity of enzyme needed to catalyze the hydrolysis of 1 mM of TA per minute under the assay conditions.

The selected isolates were identified following extraction of genomic DNA using a hermocycler. Using the electrophoresis technique, the quality of genomic DNA was ascertained by running a sample in 0.8% agarose gel followed by amplification of the 16S rRNA gene. Polymerase chain reaction (PCR) was performed using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The condition for PCR which was performed using at hermocycler (Bio-Rad T100TM, USA) with the conditions as follows: 94°C for 30s denaturation, 52°C for 30s annealing, 72°C for 90s for extension, and the final extension step at 72°C for 10 min for 30 cycles. Further, the PCR products were then purified using QIAEX gel extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The product obtained was then subjected to sequencing (Super worth Biodiscoveries Pvt. Ltd. located in New Delhi), and subsequently analyzed using the BLASTn tool available on the NCBI website (*www.ncbi.nlm.nih.gov.in/*, NCBI, USA). The sequences that closely matched were then obtained, and compared with the query sequence to determine the identity. Further, the neighbor-joining (NJ) method was used to build the phylogenetic tree using MEGA (Version 11.0; Bio design institute, Tempe, USA) between the strains that possess greater than 95% homology.

Tannase production was investigated using solid-state fermentation (SSF) with different substrates. To achieve this, a 100 ml Erlenmeyer flask containing 5 ml of Basal salt media (g/l: K_2HPO_4, 0.5g; KH_2PO_4, 0.5 g; $MgSO_4$, 2.0 g;$CaCl_2$, 1.0 g; and NH_4Cl, 3.0 g, pH 7.0) was prepared and mixed with 5 g of each substrate before sterilization at 121°C for 20 min. The

flasks were inoculated with the bacterial isolate and incubated at 37°C for 24 h. After incubation, 25 ml of acetate buffer (0.2 M; pH 5.5) was added to the medium, and the mixture was re-incubated for additional 1 h under continuous shaking at 200 rpm. The contents were separated by centrifugation at 10,000 x g for 15 min at 4°C, and the resulting clear supernatant collected for tannase activity.

Various parameters were investigated for maximal enzyme production by the selected isolate under SSF conditions. Tannase production was monitored in 250 ml Erlenmeyer flasks containing different ratios of the substrate to BSM, ranging from 1:1, 1:2, 1:3, 1:4, 1:5 and 1:6.

Similarly, different factors were studied including the inoculum percentage (1-2.5), incubation period (24-96 h), incubation temperature (25-50°C) and pH (5.0-8.0). Various carbon sources used were glucose, fructose, starch, mannitol, maltose, sucrose while supplementation of nitrogen sources such as peptone, yeast extract, beef extract, NH_4NO_3, $NaNO_3$, metal ions $CuSO_4.5H_2O$, $FeSO_4$, $MgSO_4$, $NaNO_3$, $ZnSO_4.7H_2O$, KCl, $HgNO_3$ and additives (SDS, β-mercaptoethanol, EDTA, Tween-80 and Triton-X) were included in the medium to assess its effect on enzyme production. All experiments were conducted in triplicate, and the results were presented as mean values ± standard deviation (SD).

RESULTS AND DISCUSSION

A total of 75 bacterial isolates were obtained in TA containing medium and among them 25 were randomly selected on the basis of halo on plates. One isolate was finally selected based on the higher quantity of enzyme produced. The chosen (Table 1) exhibited irregular and grayish-coloured raised colonies on nutrient agar plates, and found to be Gram-negative with small rod-shaped structures. The biochemical and physiological properties of the isolate indicated that the isolate tentatively belonged to Enterobacter genus.

PCR was performed to amplify the 16S segment of bacterial culture, and subsequent electrophoresis of the amplified products confirmed the presence of a 1.5 kb product. The consensus sequence was generated using bioedit software (*https://bioedit.software.informer.com/*) and comparison of consensus sequence was conducted using BLAST by retrieving similar sequences from NCBI data base. The data pointed out that the isolate showed closest resemblance to *Enterobacter hormaechei* subsp. xiangfangensis strain and hence inferred that the strain belonged to *Enterobacter hormaechei*. The sequence was submitted to the NCBI GenBank database under the accession number OM462375. Furthermore, the phylogenetic analysis confirmed that the strain shared homology to *Enterobacter hormaechei* as shown in Fig. 1 and confirmed to constitute a distinct clade, diverging from the other mentioned strains.

Several studies have highlighted tannase producing bacterial culture from different animal gastrointestinal tract. For instance, *Bacillus subtilis, Brevibacillus agri* and *Klebsiella variicola* were isolated from the intestinal tract of fish (Talukdar *et al.*, 2016). Similarly, different strain of *Klebsiella variicola* strain PLP G-17 LC, PLP S-18, PLP G-17 SC with tannase activity was isolated (Sharma *et al.*, 2017). Likewise, *Enterobacter cloacae, Bacillus subtilis* and *Bacillus cereus* were obtained from the intestinal tract of the Indian mole cricket (Revathi *et al.*, 2017). Other tannase producer such as *Pseudomonas* MKJP1 from the gut of silkworm *Bombyx mori* (Pandiarajan and Krishnan, 2021), *Bacillus pumilus* TT29, LB66 and LB24, *Staphylococcus warneri*, from *Acrobasis nuxvorella* (Corrales-Maldonado *et*

Table 1. Biochemical characterization of the isolate

Biochemical test	Gram's reaction	Shape	VP	MR	Catalase	Urease	Nitrate reduction	Indole production	Oxidase	Sucrose
Inference	-	Rods	+	+	+	+	+	-	-	+

Contd..

Table 1 contd.

Inference	D-sorbitol	Raffinose	Melibiose	Arabinose	Maltose	Xylose	Lactose	Mannitol	Trehalose
	-	-	-	+	+	+	+	+	+

+ Positive and - Negative.

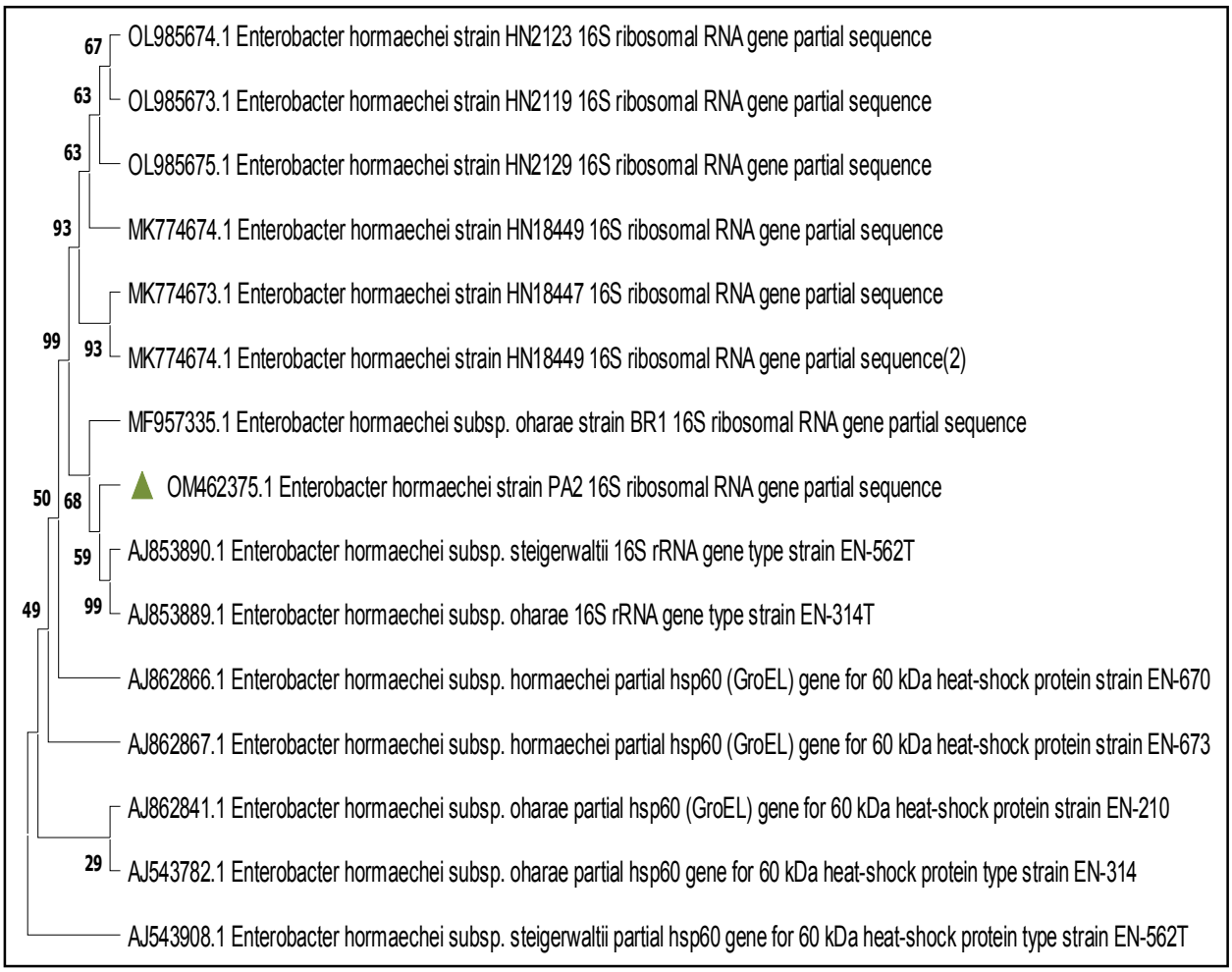

Fig. 1. Phylogenetic tree based on the nearest phylogenetic neighbors' for strain PA2 constructed with MEGA 11 by neighbor joining. The GenBank accession numbers of the reference strains are shown besides the names. The strain shows distinct linage. The significant bootstrap values are shown along with their branching position.

al., 2022) was also reported earlier. Several investigators had highlighted different enzymatic activities of microflora isolated from termite gut such as xylanase, cellulase (Cibichakravarthy *et al.*, 2017), lignocellulose, hemicellulases and pectinases (Tokuda, 2019; Sardar *et al.*, 2022). To the best of our knowledge, the present investigation for the first time underlined the characterization of *E. hormaechei* PA2 strain from termite gut that was implicated in tannin degradation.

In order to maximize tannase production, it is essential to study the optimization of various factors during fermentation reaction. Various parameters including substrate, pH and incubation temperature showed impact on the enzyme's yield and stability. Therefore, different agro waste residues were tested to identify the most suitable source under SSF conditions. Additionally, the impact of various other process parameters on tannase production was also assessed.

Various agro-waste substrates were assessed for their tannin content (mg/g dry leaves), and the results demonstrated the values e.g. 10.43, 12, 2.4, 8.07, 8.11 and 9.90 with arjun bark, betel nuts, gulmohar leaves, jamun leaves, keekar pods and tamarind seeds, respectively. Since betel nuts possessed higher tannin content, hence utilized as substrate for enzyme production. The strain displayed a significantly higher enzymatic activity of 7.47 U/ml when cultivated with betel nuts (Fig. 2), whereas the lowest enzyme titer was observed in arjun bark (2.41 U/ml). This higher activity with betel nuts may be attributed to the presence of certain factors, such as elevated tannin content and sugars, which enhance enzyme production.

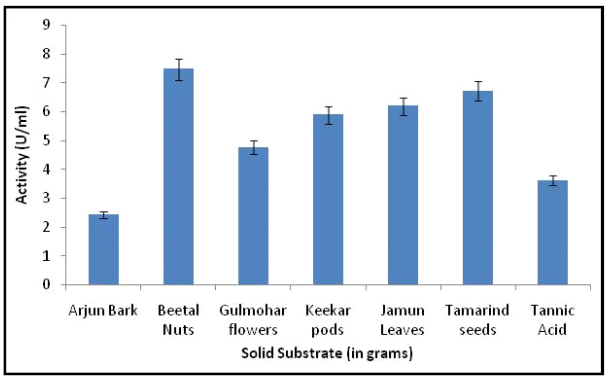

Fig. 2. Effect of substrate.

Several agro residual wastes for the production of enzymes were used as substrate. For instance, *Bacillus subtilis* isolated from the gut of fish exhibited an enzyme value 2.65 U/ml using corn substrate under solid fermentation conditions (Shakir *et al.*, 2022). Similarly, Mostafa (2022) *Penicillium commune HS2* from corn cobs demonstrated that dry potato peels as substrate was found to be responsible for maximal tannase (288.48 U /gds) production. Subbalaxmi and Murty (2016) evaluated polyurethane foam as a substrate for tannase production by *Bacillus gottheilii M2S2* isolated from soil samples of tannery effluent and proved tannase production resulted in a 3.28 fold increase under SSF. Termites are saprophytic in nature and feed on almost all tannin-containing agro waste residues, which could be a counter mechanism to decrease the toxicity of tannin in the gut environment.

The moisture content in fermentation medium during the production process affects enzyme production. The impact of moisture was assessed by varying the substrate to BSM ratio, ranging from 1:1 to 1:6. The findings revealed that a 1:1 ratio led to higher enzyme yield (3.7 U/ml), as depicted in Fig. 3 (a). Further elevating the moisture level beyond this point resulted in a reduction in enzyme production. The reduction in enzyme activity at elevated moisture level could be due to limited oxygen availability reducing spatial capacity, and alterations in particle structure causing lower biomass and decreased enzyme production. Numerous researchers have also observed moisture level in the range of 40-70% for optimal enzyme production. Shakir *et al.* (2022) concluded that 50% moisture content in fermentation was conducive for enzyme generation in *Bacillus subtilis*. Similarly, *Bacillus velezensis* TA3 possessed higher tannase activity under 55% moisture level (Lekshmi *et al.*, 2020).

A range of inoculums from 1.0 to 2.5% was used to evaluate its effect on enhancing tannase yield and it was found that 2.5% inoculum addition caused incrementing effect on enzyme production and produced 11.541 U/ml of enzyme activity (Fig. 3b). The inoculum in fermentation media played a vital role in enzyme production under fermentation conditions and lower inoculum level might not adequate for proper growth of bacteria. Shakir *et al.* (2022) also noted that 2% inoculum of *Bacillus subtilis* produced tannase enzyme in the range of 211.97±0.08 U/ml in corn leaves under SSF conditions. Similarly, *Bacillus amylolique faciens* produced higher yield of enzyme with 1% of inoculum (Shakir *et al.*, 2022), whereas *Bacillus gottheilii* M2S2 generated maximum enzymatic activity of 49.32±0.30 U/l was achieved with 4% of inoculum size (Subbalaxmi and Murty, 2016). Different incubation temperatures (25, 30, 35, 37, 40, 45 and 50°C) were used to monitor tannase production and maximum yield was observed at 37°C with 8.45 U/ml (Fig. 4a). Sheela *et al.* (2016) also reported maximum tannase yield i.e. 1.115 U/ml with the isolate

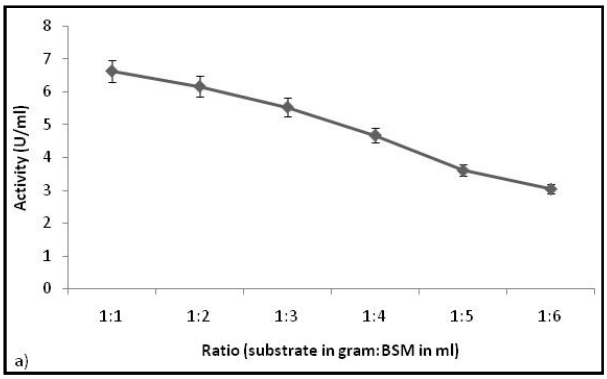

Fig. 3 (a). Effect of moisture level.

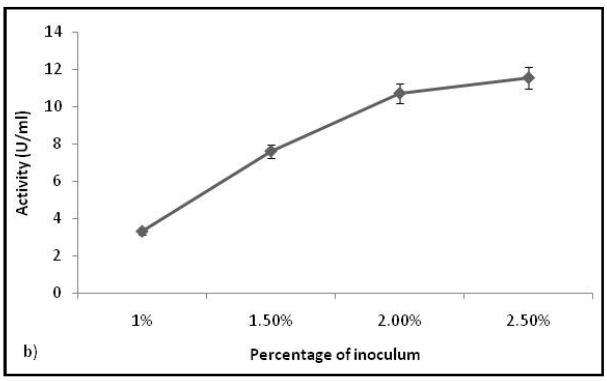

Fig. 3 (b). Effect of inoculums percentage.

Serratia marcesans at 37°C incubation temperature. Similarly, *Lactobacillus brevis* A6X, ES6 and KT2 strain yielded maximum tannase activity at 37°C (Kivanc and Temel, 2019). *Raoultella ornithinolytica* strain isolated from a gut of fish exhibited 45°C incubation temperature as the optimum temperature for the production of tannase (Shakir *et al.*, 2023). Interestingly, *Staphylococcus aureus* showed elevated tannase activity of 2.96 U/ml at 50°C incubation temperature (Isah *et al.*, 2017).

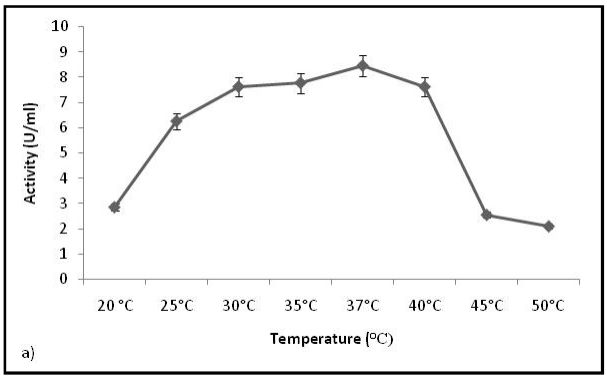

Fig. 4 (a). Effect of incubation temperature.

Tannase production was monitored for over a period of 96 h and the results revealed that higher enzyme production was achieved (7.70 U/ml) after 48 h of incubation. The enzyme activity showed a gradual declining trend up to 96-h of incubation (Fig. 4b). The bacterial isolate was likely to experience exponential growth during the initial 48 h of cultivation, thereafter; subsequent declining phase could be a rational behind lower enzyme production. Similar results were obtained in *Bacillus velezensis* TA3 that generated higher tannase enzyme after 48 h of incubation under SSF using pomegranate peel as substrate (Lekshmi *et al.*, 2020). In contrast, Shakir *et al.* (2022) reported that 24 h was the optimum time period for higher tannase enzyme yield (211.97±0.08 U/ml) using *Bacillus subtilis*. Likewise, *Bacillus amylolique faciens* showed elevated tannase activity after 24 h (Shakir *et al.*, 2022). Another bacterial strain *Bacillus gottheilii* M2S2 isolated from tannery effluent soil exhibited optimum tannase after 26.45 h under SSF conditions (Subbalaxmi and Murty, 2016).

The pH of fermentation media played a significant role in enzyme production by increasing solubility of substrates, thus

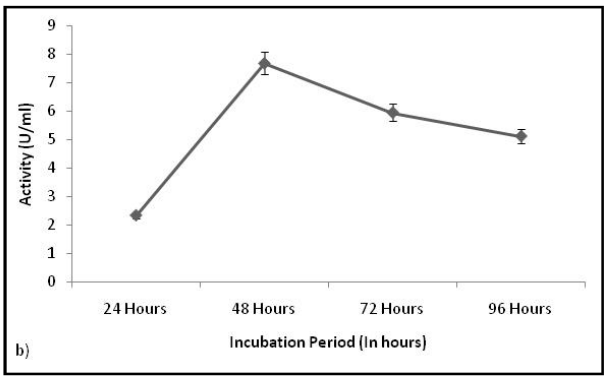

Fig. 4 (b). Effect of incubation period.

rendering it available to the organism to support the bacterial growth and metabolites production (Shakir *et al.*, 2022). A range of pH (5-8) was evaluated to study its impact on enzyme production under fermentation conditions. The data showed that the strain produced maximum tannase i.e. 4.22 U/ml at pH 7.0, whereas the further increase in pH decreased the enzyme yield (Fig. 5). The results are in accordance with several studies that reported maximal tannase production around pH 6.0 from *Serratia marcescens* (Sheela *et al.*, 2016). Subbalaxmi and Murty (2016) underlined that the *Bacillus gottheilii* M2S2 strain produced tannase at acidic pH i.e. 4.74 with tannase activity 48 U/l, whereas *E. faecalis*, *Enterococcus* sp. and *Staphylococcus* sp. proved to generate higher yield at pH 9.0 (David *et al.*, 2022).

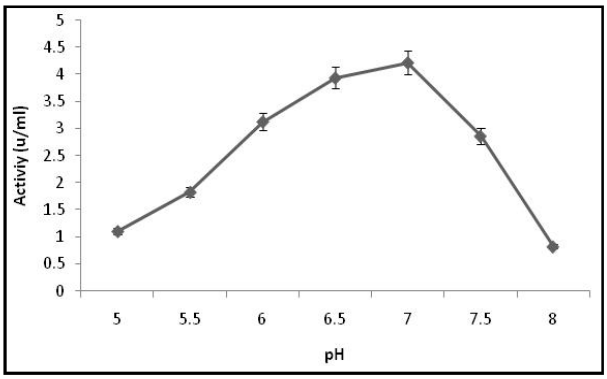

Fig. 5. Effect of pH.

The enzyme production in presence of various carbon sources including glucose, fructose, starch, mannitol, maltose and sucrose (0.2% w/v) in the fermentation media were studied and the results revealed that the highest production of enzyme was observed in maltose with the enzyme activity of 11.71 U/ml (Fig. 6a). Available reports on the influence of

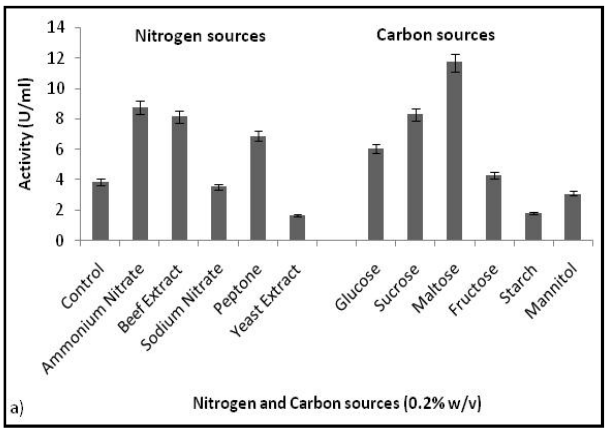

Fig. 6 (a). Effect of carbon and nitrogen sources.

carbon sources on tannase production seemed contradictory. For instance, Sheela *et al.* (2016) supported that sucrose addition in fermentation media suppressed the tannase production using *Serratia marcescens* under SSF conditions. In contrast, Lekshmi *et al.* (2020) studied various sugars impact on tannase production by *Bacillus velezensis* TA3 and contended that the addition of carbon sources did not affect the tannase activity although carbon sources inhibited the enzyme activity.

Nitrogen sources (0.2% w/v, organic and inorganic sources) like peptone, yeast extract, beef extract, ammonium nitrate and sodium nitrate were used to assess their effect on enzyme production as these played a vital role in the regulation and production of essential enzymes. The findings suggested that ammonium nitrate stimulated the enzyme production with the highest activity i.e. 8.750 U/ml when compared with other nitrogen sources (Fig. 6a). The results are in accordance with Subbalaxmi and Murty (2016) who highlighted the positive effect of NH_4NO_3 in exorbitant enzyme production by *Bacillus gottheilii* M2S2. *Bacillus subtilis* showed optimum production of tannase with supplementation of yeast extract as a nitrogen source with corn leaves powder as substrate (Shakir *et al.*, 2022). Shakir *et al.* (2023) also evidenced yeast extract as the finest nitrogen source for tannase production by *Raoultella ornithinolytica.* In another study, ammonium nitrate was found to support enzyme production under submerged fermentation conditions using *Enterobacter cloacae* 41 strain (Govindarajan *et al.*, 2019).

The evaluation of catalytic effect of different metal ions e.g. Ca^{+2}, Cu^{+2}, Hg^{+2} and sulphates of Fe^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+} (0.2% w/v) was studied and the results indicated that ferrous sulphate proved to inflate tannase production with the activity of 10.236 U/ml (Fig. 6b). Various workers underlined the stimulatory effect of metal ions on tannase production and proved the concentration of metal ions was very critical as the enzyme expressed its catalytic activity in the presence of metal ions. *Enterobacter cloacae* 41 strain showed the highest activity with Fe^{3+} metal ion but *B. subtilis* produced high tannase production with $MgSO_4$ as the most effective metal ion (Govindarajan *et al.*, 2019). Mg^{2+} gave a positive effect on tannase production by *B. subtilis* (Shakir *et al.*, 2022). Similarly, *Raoultella ornithinolytica* showed elevated level of tannase production in the presence of KH_2PO_4 through solid state fermentation (Shakir *et al.*, 2023). Evaluation of different surfactants on tannase production was studied as these play an important role in the catalytic capacity of the enzyme. The data revealed that the addition of triton x-100 in the medium enhanced the tannase production i.e. 11.918 U/ml, whereas other surfactants used such as SDS, Triton-X indicated an inhibitory effect on enzyme production (Fig. 6b). The combined effects of factors such as reduction in the hydrophobic interaction that played a role in holding the tertiary structure of protein and direct interaction with the protein molecule could be the possible mechanism behind the inhibitory action of surfactant (Nandi and Chatterjee, 2016). Major findings on surfactant effect on tannase production are either stimulatory or inhibitory. Present results are in contradiction with Borah *et al.* (2023), who found that additives like beta-mercaptoethanol inhibited

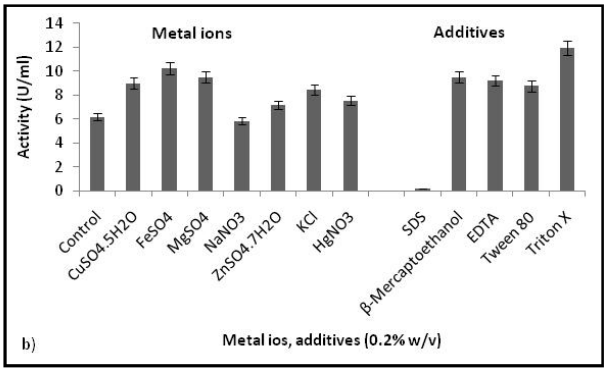

Fig. 6 (b). Effect of metal ions, additives and surfactants.

the tannase activity at higher concentrations under submerged fermentation conditions. Various surfactants used such as tween-80, DMSO and urea did not show any effect on tannase production in *Lactobacillus brevis* (Kivanc and Temel, 2019).

CONCLUSION

E. hormaechei PA2 was isolated from the termite gut, and exhibited considerable tannase activity. Different parameters were assessed for maximal enzyme production and the data showed that all factors played an important role in enhancing tannase production. Specifically, metal ions concentration, carbon source and incubation temperature significantly enhanced tannase production. The data suggested that the tannase produced within the gastric region of termites might act as a counter mechanism to mitigate the harmful effects of tannins.

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