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NEW TANNASE-PRODUCING *LACTOBACILLUS* SP. NRC10: GENE CLONING, ENZYME PURIFICATION, AND CHARACTERIZATION

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ABSTRACT

Tannins are natural polyphenolic compounds that considered nutritionally undesirable because they form water-insoluble complexes with protein, starch, and digestive enzymes and cause a reduction in the nutritional value of food and inactivate the enzymes. This study aimed to characterize an efficient tannin-degrading microorganism, able to grow under acidic conditions. Samples were collected from different places in Jeddah city. The samples were plated onto tannic acid plates containing tannic acid as the sole carbon source. The efficient tannase-producing microorganism was selected for further work. Out of 10 tannase-producing microbial isolates, the best tannase-producing, acid-tolerant bacterium was selected and identified based on morphological, physiological, and biochemical characteristics. The best tannase producer was selected and identified as a species belonging to the genus *Lactobacillus* and identified as *Lactobacillus* sp. NRC10. The results of identification were confirmed by 16S rRNA studies. Improving tannase production was carried out. The tannase –coding gene was amplified using the PCR technique. The cloning of tannase gene (Tanlp1) was carried out and its complete sequence was determined. The cloning and efficient expression of tannase gene (Tanlp1) were carried out. Precipitation and purification of the tannase enzyme in addition to factors affecting enzyme production were studied. The enzyme molecular weight was determined to be 50 KDa using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The best temperature and pH were determined to be 30°C and 6, respectively.

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Introduction

Tannase (Tannin acylhydrolase EC.3.1.1.2) is an important enzyme implicated in several science and technology fields. It catalyzes the hydrolysis of complex tannins (polyphenolic compounds). Owing to its hydrolytic properties, tannase could be used to eliminate the undesirable effects of tannins in beverages, food, feed, and tannery effluents. It can also be used for producing gallic acid, which is an impartment intermediately compound in the synthesis of antibacterial drugs [1-3]. The active components of medicinal plants are often polyphenolics. In recent years, there has been a great scientific interest for this group of compounds due to their antioxidant, antiviral, and anticancer properties [4]. Recently, tannase attracted much attention with respect to proposed applications in pharmaceuticals and in the food industry as a clarifying agent. However, industrial applications of tannase are still very limited due to its high production cost. Its commercial importance as a clarifying agent demands not only the search for better yielding viable strains but also economically viable bioprocesses for its large-scale production. Another important application of tannase is in biosensors for monitoring tannin levels in food.

Tannase can be produced by plants, animals, and microorganisms. A variety of microorganisms, including bacteria, yeast, molds, and filamentous fungi have been documented as tannase producers. The most potent producers are fungal species such as *Aspergillus* spp. and *Penicillin* spp. On an industrial scale, tannase is mainly produced by *Aspergillus* spp. [5]. A large number of bacterial species has also been reported as tannase producers, including *Streptococcus gallolyticus*[6]*Bacillus lichenifomis*,and several *Lactobacillus* species [7, 8].

The occurrence of tannase producers with respect to the environment is concerned. The majority of the tannase producing microorganisms were isolated from soil contaminated with tannery effluents. Therefore, the isolation of an efficient acid-tolerant, tannase–producer microorganism from Jeddah is highly desired for many industries and biotechnological

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applications. Optimizations of culture conditions were essential for high productivity of tannase enzymes. The cloning of tannase gene (*Tanlp*1) was carried out and its sequence was determined. Purification and characterization of the recombinant enzyme were carried out to obtain an idea about the optimum condition for the enzyme to attach tannins. The determination of the molecular weight of the enzyme was done using gel electrophoresis.

Material and Methods

Samples collection

The samples used in this study were soil, horse dung, fermented milk, and green tea. All of these samples were collected from Jeddah, Kingdom of Saudi Arabia.

Screening and selection of potential strains

One gram from each sample (soil, horse dung, or tea) was re-suspended in 20 ml of selective tannic acid medium [9] and incubated with shaking (120 rpm) at 37°C for 24h. Then, 200 µl from the previous enrichment culture was transferred to new flasks containing fresh medium [10]. After incubation, an increase in turbidity and change in cultivating medium color were observed remarking the microbial growth and the consumption of tannic acid (substrate), respectively. Isolated colonies were grown on selective tannic acid agar plates and incubated at 37°C for 48 h. Fermented milk samples were cultivated at 37°C on MRS (DIFCO, USA) agar plates supplemented with 5 g/l tannic acid (Difco, Detroit, USA). Colonies surrounded by a clear zone were selected and the isolate with the highest clear zone was chosen [9]. To 48 ml of fresh sterile STA medium in Erlenmeyer flasks (250 ml), 2 ml of the selected microbial isolate was added and all flasks were incubated at 37°C with shaking at 120 rpm on the orbital shaker incubator, for 24 h. In the case of ferment milk samples, bacterial colonies were cultivated in MRS broth supplemented with 5 g/l tannic acid. Microbial growth was determined by measuring the optical density at 550 nm using a UV spectrophotometer (Pharmacia LKB - Novaspec II, 80-2088-71). The activity of tannase enzyme was determined by using the method reported by Libuchi *et al*. [11]. All experiments were carried out in triplicate and averages were calculated.

Preservation of the microbial isolates

Bacteria, fungi, and yeast were conserved in glycerol stocks which were prepared and stored at -80°C for long-term preservation [12].

Taxonomical studies

Morphological Characterization

NRC 10 was selected as the best tannase-producing organism. It was grown on MRS agar medium for 24 h. Gram, endospore, and flagella staining tests were carried out and the preparations were examined by light microscopic using oil immersion lens. Different physiological, biochemical testes and carbohydrates fermentation were carried out according to the procedures outlined in Manual Methods for General Bacteriology [13]. Individual colonies from NRC10 were streaked on MRST plates and incubated at 37°C for 18-24 h. Oxidase and catalase production by the selected bacterial isolate were also carried out. Acidification was measured by the change in pH (Δ pH) during the incubation time. The selected isolate NRC10 was cultivated in MRS broth (pH 6) at 37°C. The pH was measured after 0.0, 4.0 and 6.0 h. using a pH-meter. The acidification values were calculated as the difference between pH at the test time and zero time (immediately after inoculation). The result showed that there was a change in pH value and Δ pH was calculated. Δ pH was ranged approximately from 0.4 to 0.8 (Δ pH = pH at test time – pH at zero time).

Molecular characterization

Phylogenetic analysis of 16S rRNA sequence

The comparison of 16S rRNA sequences is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, archaebacteria, and eucaryotic organisms. To realize this objective, genomic DNA from the selected isolate NRC10 was obtained by using QIAamp DNA Mini Kit. The primers were designed based on the highly conserved region of 16S rRNA from various bacteria. The 16S rRNA gene was amplified by PCR according to [14, 15]. The amplified PCR product of 1.449 bp was sequenced using BigDye™ Terminator Cycle Sequencing Kit. The product of the sequencing reaction was analyzed by using DNA sequencer ABI PRISM 310 genetic analyzer (Perkin Elmer, USA). Data were submitted to GenBank database. The DNA sequence was compared to the GenBank database in the National Center for Biotechnology Information (NCBI) using the BLAST program.

Optimization of the tannase production process

Microbial isolate NRC10 was grown in different media: MRS supplemented with 5 g/l tannic acid (MRST), medium I (STA) [9], medium II [16], medium III (MMT) [17] and medium X [18]. Effect of temperature (20-45°C), pH (5-9), and tannic acid concentrations, varying from 0.5%-2.5 % (w/v) on tannase production by the selected bacterium was determined using MRST broth containing tannic acid as a carbon source in 250 ml Erlenmeyer flask. Each flask containing 48 ml of the growth medium was inoculated with 2 ml of the cell suspension ($4x10^6$ cfu/ml). Growth and tannase activity were measured at the end of the growth period (2 days).

Amplification of tannase gene (*Tanlp*1)

The tannase encoding gene was amplified by PCR. Primers lp2956-f (5'-TC ATT GGC ACA AGC CAT CA-3')and lp2956-r (5'-GGT CAC AAG ATG AGT AAC CG-3') were designed and synthesized to amplify a 1419-bp DNA fragment. The

primers for the amplification of tannase encoding gene were designed based on the most conserved regions derived from the multiple sequence alignment of nucleic acid sequences encoding tannase retrieved from the GenBank (www.ncbi.nlm.nih.gov) and aligned by the Clustal W 1.83 program (www.ebi.ac.uk/clustal). The sequence of the gene, designated *Tanlp*1, consisted of 1410 nucleotides encoding a polypeptide of 469 residues. PCR reactions (100µl): 2.5 µl (20 mM) of each primer, 3µl (40 ng) of genomic DNA as a template, and 50 µl master mix with 37µl sterile distilled water. Initial template denaturation was programmed for 2 min at 95°C. Thereafter, the cycle profile was programmed as follows: 1 min at 52°C (annealing), 2 min at 72°C (extension), and 30 s at 95°C (denaturation). This profile was repeated for 30 cycles, and the final 72°C extension step was done for 7 min. The reaction mixture was subjected to a 1.0 % agarose gel electrophoresis.

Cloning of tannase gene (*Tanlp*1)

The blunt-end 1419 bp PCR fragment was extracted from agarose gel using GenJETTM Gel Extraction kit (Fermentas). The CloneJETTM PCR kit (Fermentas) was used to clone tannase-coding gene in the cloning vector pJET1.2/blunt (Fermentas). This vector contained a lethal gene that is disrupted by ligation of DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate.

The competent *E. coli* JM107 (genotype: F' *traD36 proA+B+ lacI*q Δ *(lacZ)M15* / e14– (McrA–) Δ *(lac-pro) endA1 gyrA96* (Nalr) *thi-1 hsdR17* (rk–mk+) *glnV44 relA1)* was prepared by the calcium chloride method [19]. To transform competent *E. coli* cells, up to 5 µl of the ligation mixture per 50µl of competent cells was used. The recombinant cells were plated on Luria-Bertani agar plates (LB) containing ampicillin (50 mg/ml final concentration).

Expression of tannase gene

Overnight cells pre-culture *E. coli* JM107 cells harboring the pJET 1.2/blunt/tanlp1 construct was grown in LB broth medium at 37°C with shaking at 150 rpm until the cell density OD 600 nm= 0.8. Then, the culture was harvested by centrifugation (4000 rpm at 4°C for 20 minutes). A clone containing the product insert of about 1419 bp was sent externally to sequencing after plasmid DNA isolation. The obtained sequence was analyzed by BLASTX and the translated sequence was obtained. The tannase activity was estimated as mentioned above.

Enzyme purification and characterization

The transformant *E. coli* harboring the recombinant plasmid was grown at 30°C in 2 titers of LB medium. Each flask contained 48 ml of the medium at pH 6 and supplemented with 1.5% tannic acid as a source of carbon was inoculated with 2 ml of the preculture contained $4x10^6$ CFU/ml. The culture in the stationary growth phase (after 48 h) was centrifuged at 4°C and 10,000 rpm for 10 min. The supernatant was collected and ultra-filtered using 10,000 MW cut off ultra-filter (Amicon, USA). To the ultra-filtered sample, ammonium sulfate (60 %) was added to precipitate the enzyme. The sample was then kept at 4°C for 24 h and lyophilized. The precipitate was recovered by centrifugation for 30 min at 10000 rpm and the pellet obtained was dissolved in a 20 mM acetate buffer (pH 5) and dialyzed against the same buffer at 4°C for two days. Purification was carried out using Sephadex G-200 column (2x60 cm), previously equilibrated with 20 mM acetate buffer, pH 5.0. The elution of the enzyme was carried out with the same buffer at a flow rate of 2 ml/min. Each fraction was analyzed for protein and tannase activity. The active fractions were collected and were dialyzed again against the same buffer. The active fractions were lyophilized and the concentrated enzyme (2 ml) was loaded onto an anion exchange DEAE cellulose (Sigma-Aldrich Co., USA) column (15 mm diameter and height 100 mm) pre-equilibrated with 20mM phosphate buffer, pH 7.0. The column was washed with the same buffer to remove unbound proteins. The bound proteins were eluted by applying a linear gradient of NaCl (0 to 0.5 M). The protein fractions (10 ml) were eluted at a flow rate of 1 ml/min. The pooled active fractions were dialyzed against water, concentrated by freeze-drying and stored at -20°C. In both columns, each fraction was assayed for protein (280nm) and tannase activity.

Molecular weight determination

The molecular weight of tannase was detected according to Laemmli [20] on a 0.75 mm thick polyacrylamide slab gel (7·8 cm) using mini gel system Bio-Rad. The gel was composed of 12.5% separating and 4% stacking gels. Proteins in the gel were stained with Coomassie Brilliant Blue R-250 and the molecular weight was estimated with reference to high molecular weight protein standard (Amersham Pharmacia) ranged from 25-250 KDa.

Characterization of the purified enzyme

The effect of pH on enzyme activity was studied by using various pH (5-9) using citrate buffer (0.05 M) for pH 5.0-6.0, sodium- phosphate buffer (0.05 M) for pH 6.5-7.0, and Tris HCl buffer (0.05 M) for pH 7.5–9. Tannase assay was performed as described previously [3, 21, 22].

The effect of temperature on enzyme activity was studied by incubating the reaction mixture at different temperatures ranging from 20 to 50°C and the enzyme assay was performed as described previously to determine the optimal incubation temperature [3, 21, 22].

The effect of some metal ions and inhibitors was investigated. Different metal ions like Mg^{2+}, K^{+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, chelating agent EDTA, and surfactant Tween 80 were dissolved in 0.05 M citrate buffer (pH 5) at a concentration of 1 mM. The different metal ions and agents were added to the reaction mixture before incubating, and the enzyme assay was performed as described previously [3, 21].

Statistical analysis

Each reading had three replicates. Means of variable and standard deviation were recorded. Data were subjected to statistical analysis and differences between mean values determined by the Student's *t*-test. Differences were considered significant when the probability was less than 0.05.

Results

Screening for acid-tolerant tannin-degrading microorganisms

Ten microorganisms were obtained from different samples including agriculture soil, horse dung, fermented milk, and green tea. The data are summarized in table (1). Six microbial isolates were obtained from agriculture soil (bacteria NRC 1, NRC 2, NRC 3 and NRC 4, yeast NRC 5, fungus NRC 6); two from horse dung (fungus NRC7 and bacterium NRC 8) and two isolates were isolated from green tea and fermented milk (fungus NRC9 and bacterium NRC 10, respectively). Large clear zone (+++) was observed around the colonies of yeast NRC 5 and bacterium NRC10, moderate transparent clear zone (++) was shown around the colonies of fungi NRC 6 and NRC7. No remarkable clear zone was seen around the colonies of the rest of the microbial isolates (Table 1). About four isolates out of 10 (40%) were tannase producers, of which 75% were fungi and 25% were yeast. Two microbial isolates (yeast NRC 5 and bacterium NRC 10) produced a high quantity of tannase enzyme on selective plates, whereas, low quantity of enzyme was produced by the fungi NRC 6 and NRC 7. These four isolates were selected to grow in selective tannic acid broth medium (STC) at pH 5.0 ± 0.2 and incubation was carried out at 37°C for 48h with shaking at 120 rpm. At the end of the incubation period, the microbial growth was estimated at A550 nm and the enzyme activity was determined by measuring absorbance at A380 nm in the cell-free filtrate (data not shown).

The bacterium NRC 10 isolated from fermented milk, showed a highly clear zone around the colony on selective medium (Table 1) and the highest enzyme activity (0.025±0.0102) in the cell-free filtrate. Thus, this isolate was selected for further studies.

Taxonomical studies

The isolate NRC 10 was a Gram-positive, catalase and oxidase-negative, rods shape, and aerobic bacterium. Its diameter was 0.4-0.7 and 1.4-5 µm. The organisms were capable of growing over a wide pH range (from 5.0 to 9.0). It grew at temperatures ranging from 20 to 45°C, the optimal temperature was 30°C, but they did not grow at 45°C (Table 2). It had the ability to hydrolyze starch and gelatin (Table 3). The pH range for the selectedNRC10 was 5-9 and the optimum was 6. The tested bacterium utilized different carbon sources tested (Table 4). D-glucose, fructose, D-mannitol, galactose, sucrose, and lactose were good carbon sources. The previous morphology and physiological characteristics indicated that the isolate NRC10 belongs to the genus *Lactobacillus*. Isolate characteristics were compared with data from Bergey's Manual of Systematic Bacteriology [23].

The molecular techniques were used to further confirm the identity of the strain to the species level. 16S rRNA phylogeny studies were carried out. Partial 16S rRNA was sequenced and compared to the Gen Bank database in the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) using BLASTN 2.2.6 program and Geneious v8.0.3 programs (www.geneious.com). This isolate belonged to *Lactobacillus* sp. with homology level (99.8 %) as depicted in the phylogenetic tree (Figure 1). It was identified as *Lactobacillus* sp. NRC10. The partial 16S rRNA sequence of this isolate NRC 10 showed high levels of sequence similarity with members of the genus *Lactobacillus* such as *Lactobacillus plantarum* strain BS16 (JX968493) (99.8 %), *Lactobacillus pentosus* strain Lb3F2 (KF923751) (99.8 %), *Lactobacillus plantarum* subsp. *plantarum* (AB598981) (99.8 %), and *Lactobacillus plantarum* strain JL28 (KM670021) (99.8 %).The sequence of 16S rRNA gene was determined and submitted to GenBank database under the accession number KP284304

Optimization of the tannase production process

The optimization of culture conditions for growth and tannase production by the selected bacterium NRC10 was studied by applied different media, different medium pH, different tannic acid concentration and different incubation temperatures. It was found that the growth medium affected significantly both growth and tannase production. The highest growth and the maximum enzyme activity were recorded when the bacterium NRC10 was grown in MRST medium. High growth and tannase enzyme production were also observed with (STA) medium, however, moderate tannase production and low bacterial growth were reported with (MMT) medium. Thus, it is obvious that the highest yield of tannase was obtained when the bacterium NRC10 was grown in a flask containing MRST medium, and so this medium was chosen for optimization of tannase production under different cultural and nutritional conditions (Figure 2). The effect of incubation temperature on bacterial growth and enzyme production was estimated as shown in Figure 3. The mean values depicted in figure 8 showed the distinct effect of different temperatures (20-45°C) on bacterium growth and tannase production. The maximum growth rate and tannase production were found at 30°C (0.9±0.018, 2.8±0.023) and gradually decreases with increasing incubation temperature. The optimum temperature for growth was found to be 30-40°C. In order to monitor the effect of pH on bacterium growth and tannase production, the cultivation process was carried out at an initial pH varying from 4.0–9.0. The maximum growth and tannase production were recorded. The highest enzyme production and growth were observed at pH 6 (Figure 4). Our results revealed also that maximum bacterial growth and enzyme production were obtained at pH ranged from 6-7. As an inducer, different concentrations of tannic acid were used as the sole carbon source. The results showed that the induction of tannase was obtained using 1.5 % of tannic acid as shown in Figure 5.

Sequencing of *Tanlp*1 gene from *E. coli* JM107

Twenty recombinant clones were isolated on LB supplemented with Ampicillin (50 mg/ml final concentration). Only 10 recombinants were tannase producers (selected on tannic acid agar medium). Tannase activity was estimated. The recombinant tannase producer *E. coli* was more efficient than the isolate strain NRC10. They revealed 3-fold increasing in tannase activity than that produced by *Lactobacillus* NRC10. The complete sequence of tannase coding gene was determined and aligned using Geneious 8.0.3 (www.geneious.com). An open reading frame with 1410 bp was obtained (Figure 6). The Translate predicted a sequence of 469 amino acids. The result of alignments of this open reading frame and the deduced amino acids of bacterium NRC10 with those from *Lactobacillus* spp. suggested a high level of conservation of *Lactobacillus* tannase (99.8 % homologous). Tannase from NRC10 showed about 100 % identity with *Lactobacillus pentosus* (BAN10247) and *Lactobacillus paraplantarum* (BAN81682). The sequence of *Tanlp*1 gene was determined and submitted to Gen Bank database under the accession number KP284303.

Enzyme purification and characterization

Purification of tannase was carried out using different column chromatography. High enzyme precipitation was obtained with 60% of ammonium sulfate. Further purification ultrafiltration by gel filtration chromatography with Sephadex G-200 and DEAE-cellulose yielded a high quantity of pure enzyme (Figure 7). This step eliminated proteins that might inhibit tannase activity. The purified tannase was characterized. A single peak (Figure 8) was recorded in the elution profile. A single protein band was shown on SDS-PAGE gel electrophoresis at 50 KDa (Figure 8).

The effect of incubation temperature, pH, some metal ions and inhibitors on the activity of the purified enzyme was studied. The highest enzyme activity was recorded with pH 6.0, whereas the lowest enzyme activity was reported with pH 9.0 as mentioned in Figure 9. The enzyme was stable at 30°C as shown in Figure 10. The highest enzyme activity was recorded at 30°C while the lowest was found at 50°C. A sudden drop at 50°C was observed. The effect of metal ions and inhibitors were studied after using 1mM of Ca^{2+}, Na^{+}, K^{+}, Cu^{2+}, Mn^{2+}, EDTA, and Tween 80, and the relative enzyme activity was determined. It was shown that among the metal ions, Cu^{2+} greatly inhibited the enzyme activity (60 %). When EDTA, a chelating reagent, was added to the enzyme solution at a final concentration of 1 mM, the activity of the tannase strongly inhibited (66 %). It is notable that Ca^{2+} stimulated the enzyme activity (120 %). The rest of metal ions and Tween 80 had not a significant effect on enzyme activity at a concentration of 1 mM as shown in Table 5.

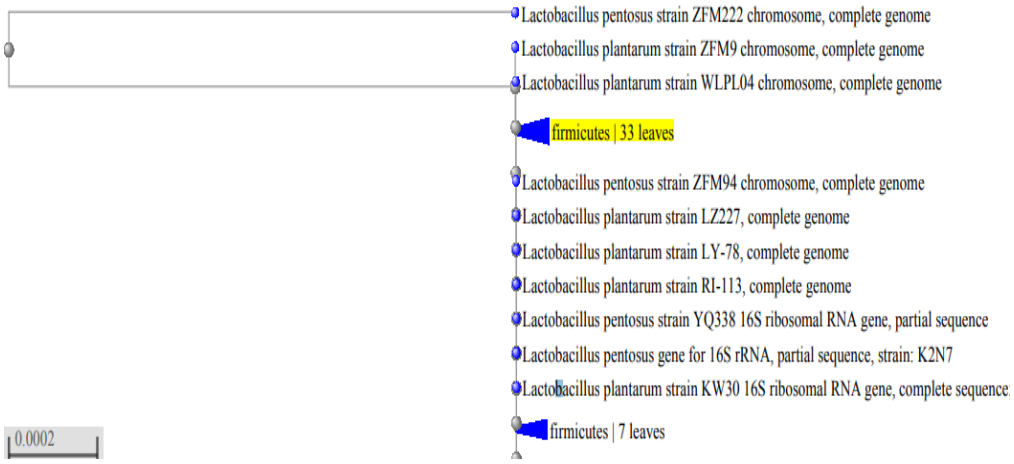

Figure 1: Phylogenetic tree based on 16S rRNA sequence comparisons of strain NRC10 and selected bacteria using neighbor-joining tree method, and maximum sequence difference =0.0003.

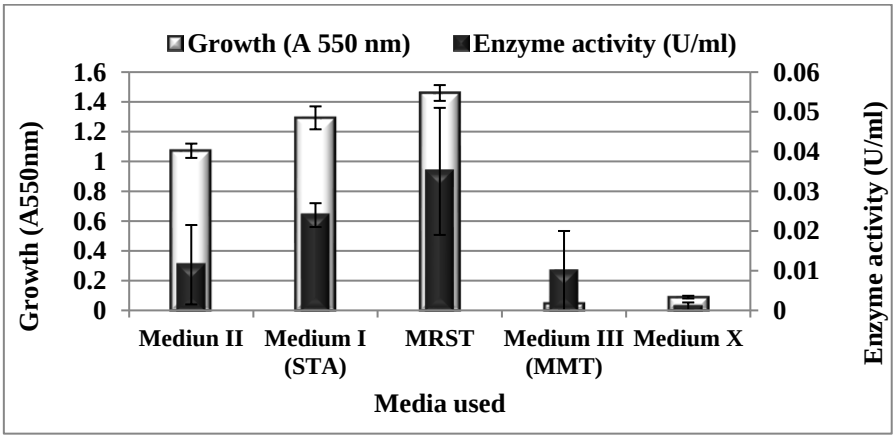

Figure 2: Effect of different media on growth and enzyme activity of the selected bacterium NRC10 after 24h.

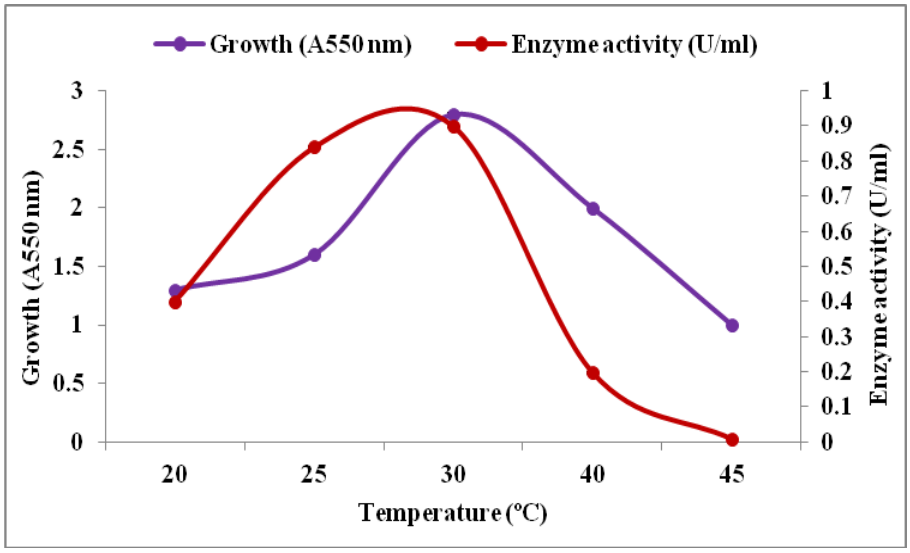

Figure 3: Effect of different incubation temperatures on growth and enzyme activity of the selected bacterium NRC10 after 24h.

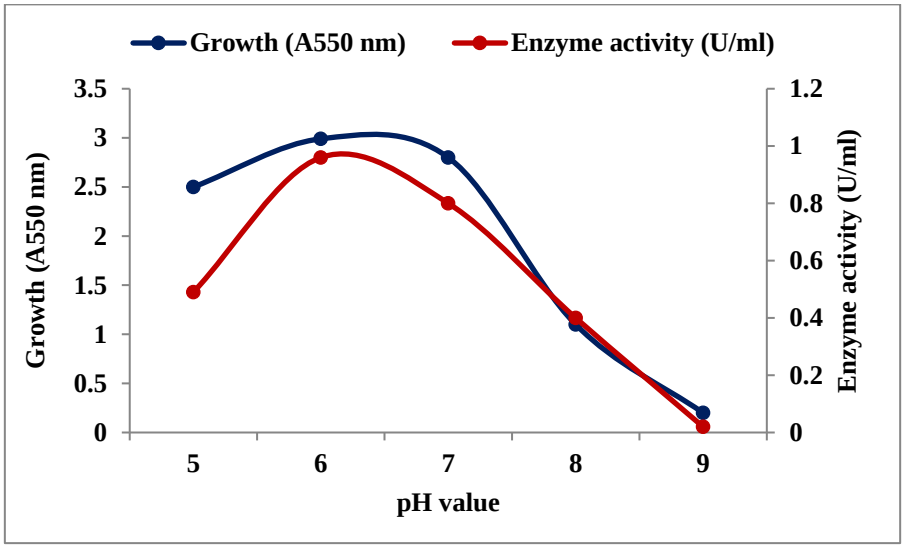

Figure 4: Effect of different pH on growth and enzyme activity of the selected bacterium NRC10 after 24h.

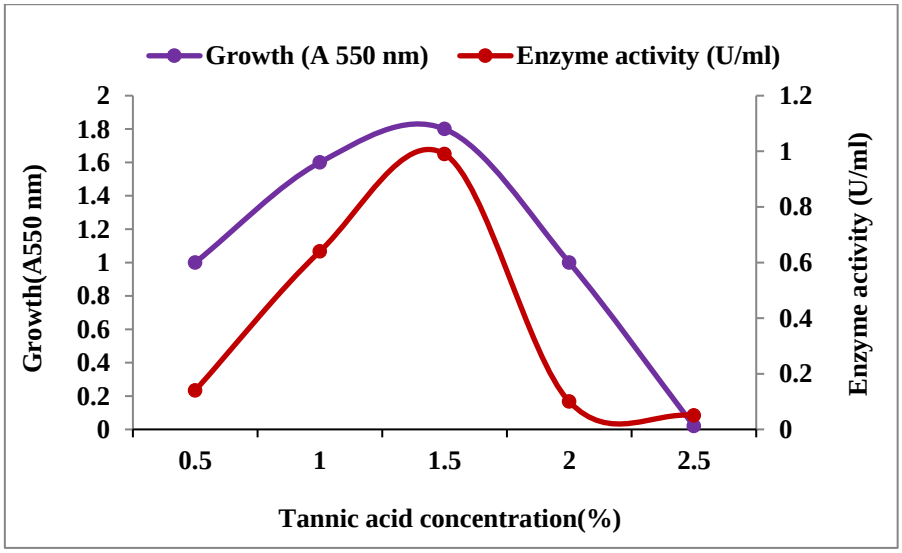

Figure 5: Effect of tannic acid concentration on growth and enzyme activity of the selected bacterium NRC10 after 24h.

Figure 6: Nucleotide sequence of *Tan1* gene and its deduced amino acid sequence.

A

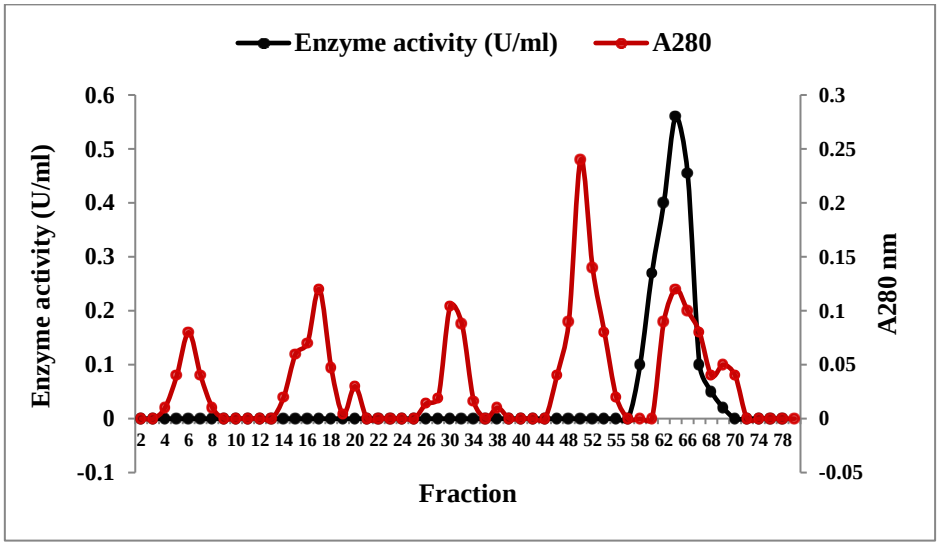

Figure 7: Elution profile of tannase from the selected bacterium NRC10 on an anion exchange DEAE cellulose column chromatography.

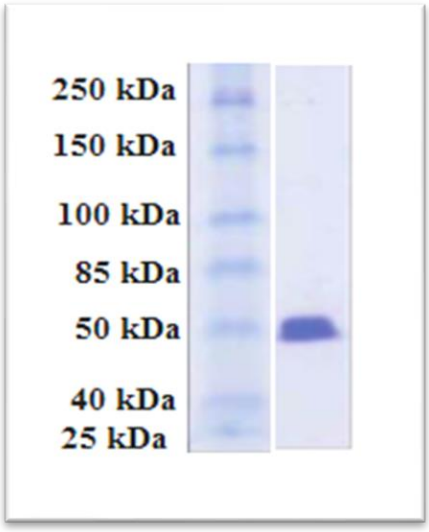

Figure 8: Molecular weight of purified tannase from selected bacteriumNRC10 was detected by SDS-PAGE. lane 1: protein marker from 250-25 kDa, lane 2: Purified tannase.

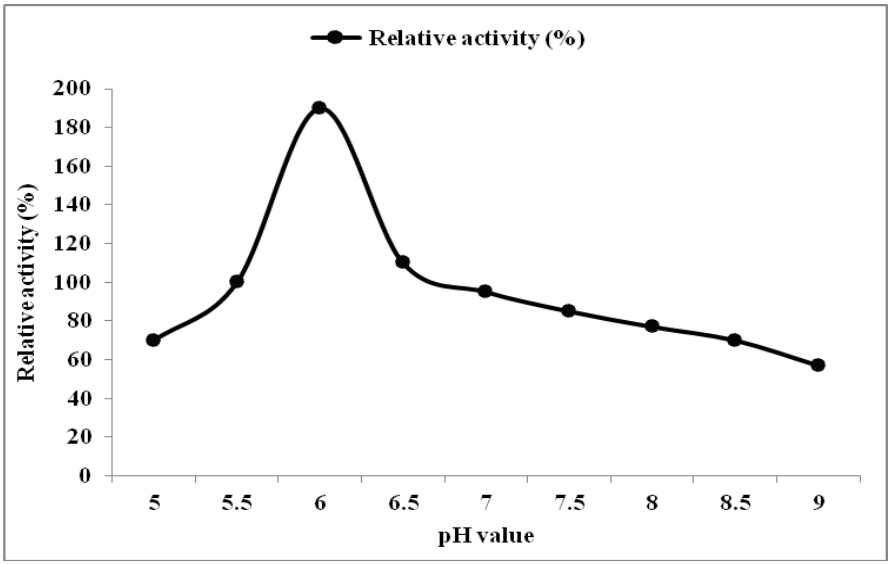

Figure 9: Effect of different pH values on tannase activity using tannic acid as the substrate.

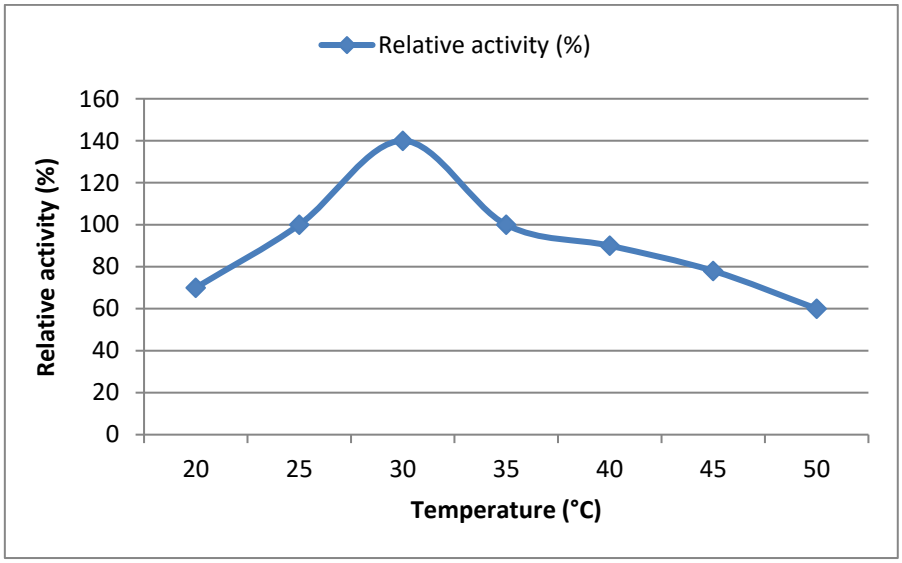

Figure 10: Effect of different incubation temperatures on tannase activity using tannic acid as the substrate.

Table 1: Growth and tannase production by isolates obtained from different sources on selective agar plates.

Microbial isolates	Source of isolation	Type of microbe	Appearance of a clear zone on selective agar medium
NRC1	Agriculture soil	Bacteria	+
NRC2	Agriculture soil	Bacteria	+
NRC 3	Agriculture soil	Bacteria	+
NRC4	Agriculture soil	Bacteria	+
NRC5	Agriculture soil	Yeast	+++
NRC6	Agriculture soil	Fungi	++
NRC7	Horse dung	Fungi	++
NRC8	Horse dung	Bacteria	+
NRC9	Green tea	Fungi	+
NRC10	Fermented milk	Bacteria	+++

+: small clear zone, ++: moderate clear zone, +++: big clear zone

Table 2: Some physiological and biochemical tests of the bacterium NRC10.

Physiological and biochemical tests	Result	Physiological and biochemical tests	Result
Gram stain	+ve	Temperature range	20-45
Spore forming	-	NaCl	0.5-5%
Motility test	-	NH_3 from arginine	+
Oxidase	-	Acid and gas from glucose	+
Catalase	-	Glucose fermentation	+
Hemolysis	-	Type of fermentation	Homo-fermentative
Lactic acid formation	+	Indole production	-

+: Present, - : Absent

Table 3: Biochemical characters of the selected bacterium NRC 10.

Substrate degradation	Results	Reaction	Results
Citrate	-	Urease	-
Na Thiosulfate	-	Oxidase	-
Amygdalin	+	Indole production	-
Tryptophan	-	Amylase	-
Na Pyruvate (VP)	-	Gelatinase	+

+: Present, - : Absent

Table 4: Utilization of different carbon sources by the selected isolate NRC10

Carbon source	Utilization	Carbon source	Utilization
Galactose	+	Raffinose	-
Glucose	+	Ribose	-
Fructose	+	Rhamnose	-
Mannitol	+	Sorbitol	-
Lactose	+	Xylose	-
Sucrose	+	Trehalose	-
Melebiose	-	Maltose	-

+: utilized, - : not utilized

Table 5: Effect of different metal ions on tannase relative enzyme activity.

Metal ions (1 mM)	Relative activity (%)	Metal ions (1 mM)	Relative activity (%)
Ca^{2+}	120	EDTA	66
Na^{+}	100	Mg^{2+}	94
K^{+}	90	Cu^{2+}	80

Discussion

In the current study, we tried to isolate and identify a new local bacterial strain, able to completely degrade tannin materials into soluble and useful materials to use in many proposes. The identification of the bacterial isolate (NRC10) was based on morphological, physiological, and biochemical characteristics. Preliminary identification tests indicate that strain NRC10 belongs to *Lactobacilli* group [12]. For species determination, 16S rRNA gene analysis was performed because distinct species may have identical 16S rRNA gene sequences due to the high diversity of members of the *Lactobacillus* species. Morphological and phylogenetic analyses assured the bacterial species determination. Finally, there was convincing morphological and molecular evidence that the isolate NRC10 belongs to the genus *Lactobacillus*.

Most of the tannase production was carried out using the submerged condition using MRST medium. High levels of tannase activity were obtained when the bacterium NRC 10 was grown for 72 h in MRST medium using 1.5 % tannic acid as a carbon source at 30°C, pH 6 with a shaking rate of 120 rpm. It is obvious that tannase production in submerged culture by *Lactobacillus* sp. was enhanced at high aeration rates and the optimum temperature and pH were reported to be 30-40°C and pH of 3.5-6.5.

The sequence of the tannase gene from *Lactobacillus* NRC10 consisted of an open reading frame of 1410 bp. The Translate predicted a sequence of 469 amino acid residues. This finding was in accordance with that found by Ueda *et al*. [24]. Recently, Ren *et al*. [25] documented a tannase crystal structure from *L. plantarum*, which possesses about 99 % amino acid identity to *Tanpl*1. These similarities are significant to distinguish the high levels of conservation of *Lactobacillus* tannase. Additionally, according to Ueda *et al*. [24], a motif consisted of Gly-X-Ser-X-Gly in *Lactobacillus* tannase amino acid sequences as the catalytic triad that displays a vital structure-function role in the binding of the enzyme to their corresponding galloyl site of the substrate, may be documented as a signature for *Lactobacillus* tannase. Indeed, this motif was not observed in the deduced amino acid sequences for *Aspergillus* tannase, suggesting a weak involvement of these amino acids in tannase function. At last, there are few sequences of genes encoding fungal and bacterial tannase described in the literature, thus this description of the sequence of the *Lactobacillus* tannase gene encoding is an essential contribution to understand the genetic information for the production of such interesting and biotechnologically valuable enzymes.

The cell-free culture broth obtained was found to be dark brown in color, indicating the possible existence of residual tannic acid in the filtrate. Treatment with aluminum oxide removed the color, leaving the enzyme crude extract colorless and transparent. The recombinant tannase from bacterium NRC10 is a monomer with a molecular mass of 50 kDa, detected by SDS-PAGE. Most known tannases are multimeric proteins, which were not in agreement with that obtained by *Lactobacillus* NRC10 in this study, supporting a new group of *Lactobacillus* tannases [24]. Monomeric enzymes have commonly been reported for bacterial tannases, like *Lactobacillus plantarum* ATCC 14917 [26]. Likewise, *A. niger* MTCC 2425 produced extracellular tannase with two different polypeptides of 102 kDa and 83 kDa, detected by SDS-PAGE [27]. Also, trimeric and tetrameric enzymes were documented [28, 29]. The molecular weight of characterized tannases was found to range from 50–320 kDa depending on the source [11, 25, 30-32].

Tannase activity was affected by several factors, especially pH values, incubation temperatures, and concentration of tannic acid. A significant increase ($p<0.005$) in tannase activity was noticed at pH 6.0 and incubation temperatures 30°C. In contrast, pH 9 and 5, and the incubation temperatures 35 and 40°C did not show any relevant effect. Many purified tannases such as *A. awamori*[33] were reported to have an optimal temperature of 35°C and maximal activity of tannase at pH 5.5. Our finding was in agreement with that obtained by Rodriguez *et al*. [34] who reported that cell-free extracts of *L. plantarum* CECT 748T (=ATCC 14917T) possessed optimal tannase activity at pH 5.0 and at 30°C. The maximum activity was found at pH ranging from 3.5 to 8.0, while optimal temperatures were from 35 to 40°C [35].

The presence of metal ions considered an important factor affecting tannase to express its full catalytic activity, so it is essential to identify the kind of ions, detergent, inhibitors and their concentrations in achieving maximal reaction efficiency. The effect of metal ions on tannase activity was recorded by Kar *et al*. [36]. One mM of divalent Mg^{2+} and Cu^{2+} did not have any significant effect on tannase activity, whereas Ca^{2+} strongly activated it. This finding was in accordance with that obtained by Curiel *et al*. [37] who reported that the activity of *TanLp*l was greatly increased in the presence of Ca^{2+}. In contrast, this finding was not in agreement with previous studies conducted by Mukherjee and Banerjee [38]. On the contrary, Mg^{2+} inhibited tannase activity from *A. niger* ATCC 16620 [3]. Mata-Gómez et al. [29] found that Cu^{2+}had a mild inhibitory effect on the enzyme activity from *A. niger* GH1. The reduction in the enzymatic activity of tannase in the presence of divalent cations may be due to nonspecific binding or aggregation of the protein, though various metal ions act as cofactors, increasing the activity of a great variety of enzymes. Other chemical substances play a role in the tannase activity as the surfactants and chelators were documented by Kar *et al*. [36]. Tween 80 had no influence on tannase activity, while 1 mM EDTA dramatically inhibited tannase activity implying that the enzyme depended on divalent metallic ions as co-factors, which is in agreement with finding reported by Mukherjee and Banerjee [38]. Also, these results were not agreed with that previously documented for *A. niger* MTCC 2425 [27] and *L. plantarum*[34] that found no significant influence of EDTA on tannase activity.

Finally, we can conclude that the extracellular tannase produced by bacterium NRC 10 showed distinctive characters, as monomeric structure and activation by Ca^{2+}, suggestive of a new kind of microbial tannase. Also, a suitable pH (6) and temperature (30°C) considered an interesting characteristic for its application in biotechnology.

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