Occurrence of a novel tannase (*tan B_{LP}*) in endophytic *Streptomyces* sp. AL1L from the leaf of *Ailanthus excelsa* Roxb

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Abstract

The tannase production ability by endophytic actinobacteria and the genetic identity of responsible tannase gene were determined. The studied strains were isolated from surface-sterilized leaf discs of *Ailanthus excelsa* Roxb. Four strains were found to hydrolyze tannic acid on solid media containing 0.4% tannic acid. The strain AL1L was found as *tan B_{LP}* indicating production of tannase with diverse of substrate affinity. The tannase production from the potential strain AL1L was performed in liquid tannic acid broth (0.4%, w/v). The strain was later identified as *Streptomyces* sp. AL1L on the basis of 16S rDNA homology. Highest enzyme activity was observed at 48 h of incubation at the exponential growth phase. The enzyme was purified by ammonium sulfate precipitation followed by dialysis (15 kD cut off). This enzyme, with molecular weight 180 kD shows highest catalytic activity at 35 °C, pH 6 with substrate concentration 0.1 g%. The purified enzyme possesses $1.4 \times 10^{-3} K_m$ and 11.15 U/ml as V_{max} . The above study indicates high industrial prospective of endophytic actinobacteria as source of tannase of potential biotechnological applications.

Keywords Endophytes · *Streptomyces* · *Ailanthus* · Tannase · *Tan B_{LP}*

Introduction

Tannins are polyphenolic secondary metabolites that are extensively produced in many species of plants, where they execute the key roles in protection from microbial attacks, pests and mites also play crucial in plant growth regulation (Banerjee and Mahapatra 2012; Thorington and Ferrell 2006). Natural tannins are either galloyl esters or their derivatives, where the galloyl moieties or their derivatives are attached to different polyol, catechin, triterpenoid or others (Khanbabaee and Ree 2001). Tannin acyl hydrolase (EC 3.1.1.20) or tannase belonging to the super-family of esterase catalyze hydrolysis of galloyl ester bond of tannins and yield gallic acid as major. After its discovery in the early 1900s, this enzyme has established wide applications

in food, feed, beverage, pharmaceutical, and chemical industries (Aguilar et al. 2007). De-esterification of tea polyphenol complexes, especially the gallated catechins are also carried out by this enzyme in preparation of instant tea (Lu et al. 2009). Tea leaves treated with tannase showed decreased level of esterified catechin content, whereas an increased non-galloylated catechin and gallic acid level. Tannase-treated tea leaves showed reduced protein-binding ability and decreased tea cream levels. The leaf extract also exhibited higher antioxidant ability than untreated tea leaves (Li et al. 2017; Liu et al. 2017). Application of tannase increases the aromatic content, maintains color, prevents any undesired turbidity and clarifies the beverage. It has high demand in homogenization of tannins in leather industry as well as in effluent treatment of water contaminated with tannins (Rodríguez-Durán et al. 2011). The gallic acid or their derivatives yielded by this enzyme also have astonishing pharmaceutical importance due to its anticancer, antimicrobial and antioxidation property (Ow and Stupans 2003). Gallic acid on the other hand is also employed in dye industries and even as photosensitive resin in semiconductor preparation (Rodríguez-Durán et al. 2011). Hence, there is an emergent interest on the basic and applied dimension of

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tannase. Though tannase can also be obtained from plants and animals, different kinds of microbes are considered as prime source at industrial level due to higher production, low expenditure and enzyme stability at wide ranges (Lekha and Lonsane 1997). In last few years, numerous attempts were made in basic and applied exploration of tannase including looking for new and novel tannase sources as it is well known that a novel source may provide some characteristically unusual molecules. Thus, there is always high interest of finding some microbes of less-explored ecosystem to get some novel metabolites.

Screening for tannases with unusual properties has long been focused on fungal source and on recombinant production (Aguilar et al. 2007; Zhong et al. 2004) with inadequacy. Recently, attention of researchers has been paid on bacterial tannase, though only few bacterial tannase are reported yet (Aguilar et al. 2007). However, to the best of our knowledge, *Staphylococcus lugdunensis* (Noguchi et al. 2007), *Lactobacillus plantarum* (Iwamoto et al. 2008; Curiel et al. 2009), and *Enterobacter* sp. (Sharma and John 2011) have been investigated for tannase from molecular genetic aspects and *L. plantarum* tannase has been characterized both biochemically and structurally (Iwamoto et al. 2008; Curiel et al. 2009; Ren et al. 2013). The tannase-encoding genes (*tan B_{LP}*, formerly called *tan_{LP1}*) (Iwamoto et al. 2008) and gallate decarboxylase (*lpdBCD*) (Jiménez et al. 2013) have been identified in *L. plantarum*.

Actinobacteria, are special group of soil-dwelling bacteria which are considered for harboring diverse of degrading enzymes. Unfortunately, till now, very few reports are available on tannase production by actinobacteria and there is no report for tannase production by endophytic actinobacteria. Endophytic microorganisms exist in plants as symptomless association. They reside in inner plant tissues without causing any immediate antagonism to the host plant. As plants are enriched in diverse types of tannins, such microbes are continuously exposed to this compound and certainly they produce tannase for their own protection as well as nutritional requirement. So there is higher opportunity to obtain tannase with altered characteristics from endophytic actinobacteria. Here, we report the presence of *tan B_{LP}* homologous novel tannase from endophytic actinobacteria isolated from leaves of *Ailanthus excelsa* Roxb. The enzyme was characterized for its genetic identity, biochemical properties, purified and analyzed.

Materials and methods

All reagents and chemicals used in present study were of analytical grade. Tannic acid, glucose and different salts used were procured from Merck Specialities Pvt. Ltd, Mumbai, India; dialysis bag, reagents used in molecular biology

were of molecular biology grade and purchased from Himedia, India; primers for PCR reactions were purchased from Sigma, USA. Any modifications of standard media have been mentioned in detail in methodology section.

Isolation of tannase-producing endophytic actinobacteria

Apparently, disease-free leaves of *Ailanthus excelsa* Roxb were collected locally and surface sterilized by the combinatorial treatment (Roy and Banerjee 2015a). Leaf discs were cut 0.5 cm diameter and placed on ISP5 agar media supplemented with cycloheximide and streptomycin (50 µg/ml). Petriplates were incubated at 28 ± 2 °C for at least 14 days. Actinomycetes coming out from the explants were immediately subcultured and preserved in glycerol stock at -20 °C. For determination of tannase production, the strains were inoculated on tannic acid agar media (tannic acid 0.4%, NH₄Cl 0.3%, K₂HPO₄ 0.05%, MgSO₄ 7H₂O 0.05%, glucose 0.001%, bacteriological agar 2%) and incubated at 30 ± 2 °C for 5 days. Appearance of transparent zones surrounding the colony indicates positive tannase production. Tannic acid-hydrolyzing efficiency of each isolates was determined using the following formula:

$$\text{Hydrolyzing percentage (\%)} = \frac{\pi r_1^2 - \pi r_2^2}{\pi r_2^2} \times 100.$$

Here r_1 is the diameter of hydrolyzed zone including colony and r_2 is the actinobacteria colony diameter. Unit activity = 10% hydrolyzing ability.

Amplification of *tan A_{LP}* and *tan B_{LP}* genes

Genomic DNA was isolated from the 48-h grown culture of ALIL after treating with lysozyme (10 mg/ml) and SDS (10%, w/v). Genomic DNA was extracted with phenol: chloroform: isoamyl alcohol and precipitated with isopropanol (Roy and Banerjee 2015b). Finally, it was taken in TE buffer (pH 8) and directly used as template for specific PCR amplification of tannase-specific genes. The *tan A_{LP}* and *tan B_{LP}* genes were amplified using primers 951 (5' TGATGCTGACTGGCT GGTGC) and 952 (5' GCACAAGCCATCAATCCAGG); 953 (5' CCTGATGAGTGGTTTGTAG) and 954 (5'-CTTGCGTTCTGCTTCGGTATG), respectively (Jiménez et al. 2014). PCR reaction mixture contained 2X PCR Taq mix—12.5 µl; each primer—2 µl; template DNA—5 µl and nuclease-free water—5.5 µl. Reaction conditions were as follows: initial denaturation at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and primer extension at 72 °C for 30 s. A final extension was done at 72 °C for 10 min. Amplified products were analyzed after PCR reaction on agarose gel (2%, w/v) with standard marker.

Identification of endophytic actinobacteria

The most efficient tannase-producing isolate AL1L was characterized by determining their cellular morphology, spore orientation if any, and colony characters (Williams et al. 1983). Other extracellular enzyme production by this strain was also recorded (Gordon et al. 1974). Electron microscopy of strain AL1L was done after solvent evaporation in vacuum and taken up to critical point drying. To minimize sample charging, the dried samples were coated with thin gold layer right before electron microscopy. Samples were analyzed using a Zeiss EVO 18 scanning electron microscope (SEM) with an accelerating voltage of 5 kV.

For molecular identification, genomic DNA was used as template for specific PCR amplification of 16S r DNA with primers; 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-GGTACCTTGTACGACTT-3'). The PCR product was purified with Hi-PurA. TM. PCR Product Purification Spin Kit (Himedia Laboratories, India) according to manufacturer's instructions. Amplicons were analyzed using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The sequence obtained was queried against NCBI/GenBank database (nr database). Based on the maximum identity, sequences were selected and aligned using the multiple alignment software, ClustalW. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). Bootstrap analysis with 1000 replicates was performed to estimate the confidence of a particular clade. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1393 positions in the final dataset. Evolutionary analyses were conducted with MEGA7 (Kumar et al. 2016).

Determination of tannase activity

Extracellular tannase production by strain AL1L was determined following the standard method of Banerjee and Pati (2007) with few modifications. Production was carried out for 5 days in tannic acid broth at pH 6 in 30 °C under shaking speed, 150 rpm. Enzyme activity of cell-free culture media was checked and turbidity of media was measured with respect to uninoculated same media each day. Enzyme assay was performed with 0.1% tannic acid (150 µl) as substrate with culture supernatant (50 µl) of each variant. Enzyme–substrate mixture was incubated at 30 °C for 2 h, after which 1 ml of chilled BSA (0.5 mg/ml) was added and centrifuged at 5000 rpm for 15 min. After discarding the supernatant, residual pellet was dissolved in SDS-triethanolamine and 500 µl of freshly prepared acidic FeCl₃ solution. The mixture was left for 30 min and finally OD values were

taken at 550 nm with UV–Vis Spectrophotometer (UV-1800, SHIMADZU). Control experiment was performed where distilled water was used instead of cell free culture broth as enzyme source.

Liquid fermentation for tannase from strain AL1L

Selected strains were grown on ISP5 agar till spore formation, after which 1 cm² (surface area) agar block was transferred in 30 ml of seed generation media. The composition (mg/100 ml) of seed media was tannic acid 400; NH₄Cl 300; K₂HPO₄ 50; MgSO₄·7H₂O 50 and glucose 10. The culture was incubated at 30 ± 2 °C for 18 h under a shaker incubator at 150 rpm and used as seed for final fermentation. The 5% (v/v) inoculum was transferred to final tannase production media containing (mg/100 ml) tannic acid 400; NH₄Cl 300; K₂HPO₄ 50; KNO₃ 100; MgSO₄·7H₂O 50 and glucose 1. Fermentation was carried out at the same conditions for 2 days. Enzyme activity was determined at each day of incubation.

Partial purification and PAGE analysis of enzyme

Cell masses were separated from fermented broth by Whatman filter paper and filtrate was centrifuged at 10,000 rpm for 15 min. The supernatant was then taken for graded ammonium sulfate precipitation of proteins. The 70% ammonium sulfate-saturated precipitation was centrifuged at 10,000 rpm for 5 min and the pellet was dissolved in 3 ml of acetate buffer (0.1 M, pH 5.6). The fraction was dialyzed (Dialysis Membrane-150, HiMedia, Mumbai, India). Thus, purified enzyme was determined for tannase activity by modified tannase assay method as described earlier. The purified sample (30 µl) was analyzed through 12% poly acrylamide SDS gel electrophoresis without β-mercapto ethanol and boiling. After complete run, the protein band was developed by silver staining and compared against the marker bands.

Characterization of the enzyme

Various physical and chemical factors that may influence the tannase activity were evaluated to determine optimum conditions for highest enzyme activity. For this purpose, incubation temperature, pH of reaction mixture and the substrate concentration were considered as prime input variables and their optimum values were determined by one-variable-at-a-time approach. Enzyme–substrate reactions were performed at different temperature conditions (25, 30, 35, 40 and 50 °C) and different pH conditions (3.6, 4, 4.6, 5, 5.6, 6 and 6.6) with 0.1% tannic acid as substrate. The pH of reaction mixture was maintained at 5.6 during temperature optimization and temperature was maintained at 35 °C (pre-optimized

value) during pH optimization. Optimum substrate concentration was determined by performing enzyme–substrate reaction at graded substrate concentrations (0.001, 0.005, 0.01, 0.05, 0.1, 0.125, 0.15 g%) under optimum temperature and pH values. The K_m and V_{max} of the purified tannase were also determined from the unit activities at different substrate concentrations.

Results

Tannase producibility of isolates and characterizations

In this study, ten endophytic actinomycetes were isolated and maintained in 30% glycerol at $-20\text{ }^{\circ}\text{C}$. All isolates were evaluated for extracellular tannase production. Tannic acid hydrolysis was observed as clear zone surrounding the actinobacteria colony. Strain AL1L and AL3L showed almost similar tannic acid-hydrolyzing efficiency on solid media, whereas the strain AL7L and AL8L were less active as tannase producer (Table 1). Extra cellular tannase activity by strain AL1L (6.96 U/ml of crude enzyme) was found comparatively higher than any other strains isolated (Fig. 2). It was also noticed that highest enzyme activity was achieved at 48 h of incubation, whereas increasing bacterial growth observed beyond that period (Fig. 1). Though growth rate was declining after 48 h, the bacterium was still growing at comparatively lower rate that was represented by increasing optical density of growth media up to 196 h.

Description of strain AL1L

Morphological and biochemical studies revealed that strain AL1L is Gram positive, less-branched spore-bearing actinobacteria. The strain produces chalky white to gray aerial

mycelia and substrate mycelia were gray on ISP5 agar after a 2-week incubation. Smooth cylindrical spores were observed under SEM observation (Fig. 2). The taxonomic identification was made of the strain AL1L based on 16S rDNA sequence analysis. The sequences were deposited in NCBI GenBank. Approximately, 1500 bases of the amplified product were sequenced, and homology was determined in NCBI database. The constructed phylogenetic tree revealed that the strain showed close sequence similarity (99% similarity with 98% query coverage and E value 0) with different type strains of *Streptomyces* (Fig. 3). Based on the cultural, morphological, physiological, and sequence correlation strain, AL1L was identified as *Streptomyces* sp. AL1L.

Occurrence of *tan B_{LP}* gene among selected strains

To know the extent of bacteria-specific tannase genes, presence of the *tan A_{LP}* and *tan B_{LP}* were studied within potential tannase-producing endophytic actinobacteria. An amplified PCR product of DNA (1 kb) was obtained using *tan B_{LP}* oligonucleotide from strain AL1L only.

Purification and characterization of enzyme

A total of 500 ml of liquid media was employed for tannase production by strain AL1L. Culture filtrate was obtained as 467 ml after cell separation. Enzyme activity of this crude sample was found 6.96 U/ml, whereas the relative activity has been increased about twofold in case of purified sample (15.58 U/ml). The molecular weight of purified protein enzyme was revealed as 180 kD in non-denatured/non-reducing environment (Fig. 4). The purified enzyme was found to have K_m (1.4×10^{-3}) and maximum velocity of reaction (15.6 U/ml). The tannase produced by this endophytic actinobacteria was found to retain its catalytic property between pH 5.5–6.5 though best at pH 6. The optimum temperature was found at $35\text{ }^{\circ}\text{C}$ with a range of $30\text{--}40\text{ }^{\circ}\text{C}$ (Fig. 1). The enzyme activity was found sharply increasing with substrate concentration up to 10 mg/ml of tannic acid used as substrate in this experiment. Highest catalytic activity was achieved with 100 mg/ml tannic acid as substrate concentration.

Discussion

Diverse of microbes including filamentous fungi of the *Aspergillus*, *Penicillium* genus and bacteria of the *Bacillus* and *Lactobacillus* genus have been meticulously scrutinized for tannase production since last few decades but very little documentations are available on tannase production by actinobacteria (Aguilar et al. 2007). Presently, endophytic actinobacteria have been exploited extensively

Table 1 Hydrolyzing efficiency of endophytic actinobacteria isolated from *Ailanthus* leaf

Strain Name	Hydrolysing efficiency ^a (unit)
AL1L	37.25
AL2L	—
AL3L	35.6
AL4L	—
AL5L	—
AL6L	—
AL7L	11.6
AL8L	16.6
AL9L	—
AL10L	—

^aValues represent mean of three replicates

Fig. 1 Characterization of tannase produced by strain AL1L; **a** comparative tannase activity of for tannase producers including growth pattern of strain AL1L, **b** optimum temperature, **c** optimum pH and

d optimum substrate concentration for highest tannase activity produced by strain AL1L

Fig. 2 Scanning electron micrograph of *Streptomyces* sp. AL1L showing less branched smooth surface cylindrical mycelia

for the production of various bioactive substances including industrially important enzymes. Unfortunately, such special group of prokaryotes is not yet significantly reported for

their tannase production ability and even no genetic characterization for this protein is accessible from endophytic microbes.

This study shows that 40% of the isolated endophytic actinobacteria were able to utilize tannic acid as sole carbon source. Tannic acid-hydrolyzing ability was found highest for strain AL1L which was identified as *Streptomyces* sp. AL1L. The Evolutionary analyses indicates strain AL1L that shows close relationship with *Streptomyces lienomycini* FoSt11 (KM370032.1) and several other type strains *Streptomyces* sp. HN40, *Streptomyces* sp. HN25 and *Streptomyces* sp. B425. Endophytes residing in plant tissues containing plenty of natural tannins. Thus, chances of tannase production by associated microorganisms are higher. Tannins protect plants from intruder microbes due to its phenolic nature. Thus, here it can be assumed that somehow such microbes escape from tannin toxicity and they must strictly regulate their tannase production at a level that will not cause any dilemma to their host to fight against pathogens. As actinobacteria are well known for various degradative enzyme production and being endophytic are constantly exposed

Fig. 3 Neighbor-joining phylogenetic tree based on 16S rRNA sequences (1497 bases), showing the relationship between of strain AL1L with other close relatives of the genus *Streptomyces*. Bar indicates 0.002 nucleotide substitutions per site

Fig. 4 SDS-PAGE analysis of purified protein; (M) molecular weight marker, (1) and (2) purified tannase

to plant tannins, so there exist a higher opportunity to find tannase-producing endophytic actinobacteria with unusual properties.

Screening of new tannases with altered properties has long been focused on fungi (Zhong et al. 2004). Recently, there has been increasing interest in characterization of tannase genes from bacteria upon the availability of the large amount of genomic data accumulated, although only a few bacterial tannase genes have been identified. The best characterized bacterial tannase is that from *L. plantarum* (Lp-Tan). *L. plantarum* is a lactic acid bacteria, most frequently encountered in the fermentation of plant materials where tannins are abundant. Until now, no tannases have been characterized genetically at the molecular level from endophytic actinobacteria which may provide an opportunity to isolate new enzymes with novel properties. Present study reports occurrence of *L. plantarum* gene-encoding tannase (*tan B_{LP}*, formerly called *tan_{LP1}*) homolog within a tannic acid-hydrolyzing endophytic actinobacteria. Such tannase has greater industrial application than any other type of tannase. The *tan A_{LP}* has a specific activity ten times lower than the specific activity for *tan B_{LP}* tannase and beside that *tan B_{LP}* can hydrolyze almost all kinds of tannins (Jiménez et al. 2014). Tan A gene was amplified from *Staphylococcus lugdunensis* MTCC 3614 (Microbial Type Culture Collection) and cloned in *E. coli* DH5α. The enzyme was purified from recombinant strain and found with approximate molecular weight of 66 kDa (Chaitanyakumar and Anbalagan 2016).

Strain AL1L was selected for farther study due to *tan B_{LP}* encoded tannase production and higher tannase activity. It produces tannase that have molecular weight of about 180 kD in native state. Microbial tannases are

found to be homomultimeric (Sivashanmugam and Jayaraman, 2011) though Ramirez-Coronel et al. (2003) isolated a thermo stable tannase present in monomeric and dimeric forms with molecular masses of 90 and 180 kD from *Aspergillus niger*. Tannase produced from different fungi are reported generally with larger molecular weight (70–180 kD) and exist in hetero- or homo-oligomeric form having two to eight subunits (Aguilar et al. 2007; Hatamoto et al. 1996; Zhong et al. 2004). Moreover tannases isolated from different *Aspergillus niger* are documented of having a molecular weight around 150–350 kD (Farias et al. 1994). On the contrary, bacterial tannase has been reported to possess comparatively lower molecular weight (Rodríguez-Durán et al. 2011). An Ss-Tan/tannase (Lp-Tan homolog) from *Streptomyces sviveus* was produced in *E. coli* revealed molecular weight of 54 kD (Wu et al. 2015). Enzymatic assay revealed that the protein could hydrolyze both the ester and depside bonds in hydrolyzable tannins with higher preference of substrate affinity and catalysis to depsides. In our study, it was found a 180 kD tannase with *tan B_{LP}* homology within endophytic *Streptomyces* sp. AL1L, whereas the first cloned tan LPI was found with molecular weight 50 kD containing 1410 bp, encoded 469 amino acids with 28.8% sequence similarity to TanASI from *S. lugdunensis* (Iwamoto et al. 2008). Kinetic parameters of this enzyme were measured employing tannic acid as substrate. This tannase has lower K_m value which indicate a higher substrate affinity as evidenced by K_m ; 1.4×10^{-3} which indicates its future potential to be used for commercial purpose as compared to other fungal tannase with comparatively lower substrate affinity (Aguilar et al. 2007; Chae and Yu 1983).

Plants contain various tannins and to use it for the nutritional purpose *tan B_{LP}* tannase is more advantageous as it can hydrolyse various ester bonds. Thus, this gene has been naturally selected and become evolutionarily prevalent among tannase-producing bacteria as was found in our study also. The optimum plant physiological temperature and pH retains in between 25 and 30 °C and 6.5–7.5, respectively. Endophytes live in such conditions and their metabolic status could be partially attributed to the environment they reside. The tannase obtained from this endophytic actinobacteria indicated the optimum tannic acid catabolism at 35 °C and pH 6. Most of fungal or bacterial tannase reported so far shows slightly acidophilic in nature similar to this tannase, while temperature optima ranges from 35 to 40 °C have been reported (Farias et al. 1994). Tannase (*tan B_{LP}*) from *L. plantarum* is known to possess optimal temperature and pH for 40 °C and 7–8. So, it might be assumed that during co-evolution and by the pressure of natural selection, this endophytic actinobacteria has somehow modified its protein either in genomic or proteomic level for better adaptation in that specialized micro-ecosystem.

Conclusion

This is the first report on extracellular *tan B_{LP}* tannase production from any endophytic *Streptomyces*. This tannase possess higher molecular weight of about 180 kD in native state. The present paper also states about the molecular and kinetic parameters of this tannase that significantly differs from the similar tannase produced by *L. plantarum* in terms of optimum pH and temperature. Occurrence of *tan B_{LP}* tannase among endophytic isolate *Streptomyces* sp. AL1L with some altered catalytic properties certainly aid in adaptive mechanisms of endophytes with their hosts. However, such finding is very important in basic science of host-endophyte interactions as well as for industrial exploitation of endophytic *Streptomyces*.

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