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Bacterial Degradation of Bacteriostatic Polyphenols, Tannins

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ABSTRACT

Tannin degradation by bacteria has not been studied much as tannins are commonly known to be bacteriostatic due to enzyme inhibition, substrate deprivation, and the enzyme activity on the bacterial cell wall. However, about a handful of bacteria have been found to tolerate certain concentrations of tannin. This study focuses on isolating and identifying bacteria from decaying portions of tree bark for tannase production and effective catalysis of ester bond hydrolysis in tannins. Different concentrations of commercial tannic acid were used as the sole carbon source on mineral salt medium (MSM) agar plates, to test the maximum tolerable concentrations (MTCs) by the isolates. Tannin degradation was confirmed by a visual reading method and bacterial tannase activity and the biodegradation percentage were determined. One particular isolate was identified to have 50 g/L MTC of tannin, with a tannase activity of 51.61 U/mL that is optimum after 96 hours of incubation. The 16s rRNA sequencing results showed that the isolate belonged to *Bacillus* genus and the resulting bacterial strain isolate was found to be a new strain of *Bacillus subtilis* which was submitted to GenBank under the accession number MH330408.

1. Introduction

Tannins are complex recalcitrant compounds that are consumed by humans on a regular basis as they are universally present in plants and represent the fourth most abundant group of secondary metabolites after cellulose, hemicellulose and lignins [1]. These are naturally occurring water soluble polyphenols that are found in many parts of plants such as leaves [2], fruits [3], food grains [4], bark and wood etc., [5]. Moreover, they are unique among their contemporaries as they precipitate proteins, minerals, starch, cellulose etc. from solutions [6]. Tannins in other words are water-soluble polyphenols with a molecular mass ranging from 0.3 – 5 kD that are known to be inhibitory against several organisms [7] and are recalcitrant to biodegradation [8]. Their role primarily, has been studied to guard the vulnerable parts of plants from microbial attack by inactivating the invasive microbial extracellular enzymes [9].

As per the studies conducted, continuous exposure to tannins can cause potential health hazards such as damage to the eye, skin, respiratory, and gastrointestinal tract [10]. The usual complexation of proteins to form water insoluble complexes with tannic acid and naturally occurring tannins inactivates the enzymes [11]. Therefore, foods rich in tannins are of low nutritional value as they form irreversible complexes with proteins, starch and digestive enzymes [12].

A certain group of tannins called hydrolysable tannins (e.g. tannic acid) are toxic to animals and cause poisoning if consumed by them in large amounts [13]. While on the other hand, another group called condensed tannins, also known as proanthocyanidins have many health promoting potentials, such as anticancer, antioxidant, anti-inflammatory, anti-asthmatic, and antimicrobial properties [14]. However, the large amount of tannins in foods is responsible for its unpleasant taste and an astringent feel [15] and its prolonged exposure may even bring about liver damage and other health complications.

Bearing in mind the adverse health effects caused by these polyphenols, it is necessary to degrade these harmful compounds for their successive elimination from the body. Some methods have been explored to remove tannins, including physical, chemical, biological and other methods. For degradation by microbes, tannase, the hydrolase group of enzymes is solely responsible for tannin degradation and in suppressing its anti-

nutritional activities. In other words, it is a universal enzyme that catalyzes the first step of tannin degradation [2].

Tannase is used extensively in pharmaceuticals, food processing, brewing, textiles, detergents and tea industry [16]. However, its applications are limited due to the enzyme being secreted in extremely low amounts causing inadequate substrate binding spectrum and increased cost of purification. Apart from degrading tannic acid, bacterial tannases have also been studied to degrade natural tannins like chestnut and oak tannins, as stated by Lewis and Starkey [17]. Similarly, fungal tannases are very diverse and efficiently degrade different types of hydrolysable tannins [18]. And even though a number of tannins degrading microorganisms have been reported as according to Bhat *et al.* [3], prolific degraders of tannins and better sources of tannase are still on the lookout.

Recent findings indicate that most of the organisms capable of degrading tannins isolated till date are either anaerobic or facultatively anaerobic bacteria from the alimentary canal of ruminating animals or fungal strains associated with the degradation of wood and forest litter [1]. A major problem studied in the utilization of fungal strains for industrial applications was that degradation by fungi is relatively slow and also that it is difficult to manipulate fungal strains genetically because of their complexity. And although several tannin degrading anaerobic bacteria have been isolated [19, 20], the processes based on anaerobic bacteria are slow, and the study of tannin degradation pathway is relatively difficult, which demands for a better and faster tannin degrader.

Although tannase is present in plants, animals, and microorganisms, it is mainly produced by microorganisms [21,22]. In view of this, the aim of the present study was to isolate from decaying bark samples some highly sensitive bacteria, preferably new or lesser-known strains with high tannase activity. The bacterial strain degrading tannic acid most effectively was characterized and identified on the basis of 16S rRNA sequencing.

2. Experimental Methods

2.1 Collection and Sample Preparation

The decaying bark samples were randomly collected in a sterilized petri dish, from a total of seven trees in Ayanavaram, Chennai. The collected bark samples were transferred into a conical flask containing 100 mL of MSM (mineral salts medium) broth and was homogenized by keeping it in an orbital shaker at 37 °C for 72 hours.

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2.1.1 Composition of MSM Supplemented with 3% Agar

Tannic acid (2 g/L), KH_2PO_4 (3 g/L); $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (6 g/L); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g/L); yeast extract (8 g/L); NaCl (5 g/L); Agar (3 g/100 mL).

2.2 Isolation of Tannase Producing Microbes

MSM Agar was used to cultivate microorganisms and plating was done by serial dilution method [23]. The serially diluted cultures were streaked onto sterile nutrient agar plates containing 2% tannic acid and the colonies were allowed to develop by incubating the plates at 37 °C for 24 hours. The isolates obtained were separately streaked and were then maintained on MSM agar slants supplemented with 1% tannic acid as the sole carbon source. The isolated strains were sub-cultured periodically, and the stock cultures were maintained at 30 °C. The microbial isolates were then tested for their ability to hydrolyze tannin to gallic acid and glucose by the enzyme tannin acyl hydrolase.

2.3 Identification and Characterization of the Isolated Bacteria

The isolates obtained was identified and characterized based on morphological and biochemical tests. Gram staining was performed for morphological identification followed by colony characterization. Biochemical tests such as methyl red test, Voges Proskauer test and indole test were performed. Further, the following assays were conducted to check the tannase activity of the isolate.

2.4 Tannase Activity

2.4.1 Maximum Tolerable Concentration

The maximum tolerable concentration (MTC) of tannins was studied for the highest concentration of tannic acid that allows growth after 24- 48 hrs. The increasing concentration of tannic acid (1, 5, 10, 20, 30, 40, 50 g/L) on MSM agar plates were used for testing the MTCs [24].

2.4.2 Visual Detection Assay

Isolation of tannase-producing bacteria was carried out as per the method of Osawa and Walsh [25] and our newly developed method. The inoculum suspension was poured into wells made on nutrient agar plates supplemented with tannic acid (0.1%; filter sterilized). Further, in our newly developed method, a liquid inoculum suspension of MSM broth supplemented with 1% tannic acid was prepared to allow release of the tannase enzyme by the isolates in separate conical flasks. Crude enzyme containing inoculum that had been incubated for up to 72 hours was then directly poured into wells created on purely agar containing plates, with tannic acid added as the substrate for enzyme activity. Addition of tannic acid to nutrient agar forms a tannin protein complex; cleavage of this complex by bacteria producing tannase forms a dark zone of clearance.

2.4.3 Tannase Specific Assay

This is a colorimetric method studied by Brahmabhatt and Modi [26] where tannase breaks tannic acid which acts as a substrate, forming gallic acid and liberating glucose. Tannase activity was determined by estimating the reduced glucose liberated using 3, 5-dinitrosalicylic acid reagent. One unit of tannase activity was defined as the amount of enzyme releasing $1 \mu\text{mol min}^{-1}$ of glucose under assay conditions.

Standard curve was studied by preparing dilutions up to 1000 $\mu\text{g/mL}$ of glucose solution. The activity was determined by using the supernatant or filtered cell-free broth obtained after centrifugation at 8000 rpm at 20 °C for 15 min and pre-incubating it with 0.1 M acetate buffer (pH 5.0) containing 0.5% tannic acid as substrate. 1 mL of crude enzyme was reacted with the equal amount of substrate, dissolved in the buffer and incubated for 30 min at 37 °C followed by the incubation in boiling water bath for another 15 min to deactivate the enzyme-substrate activity. From this 2 mL system, 1 mL was withdrawn and reacted with 3,5-dinitrosalicylic acid reagent and finally the system was diluted by adding 10 mL distilled water and absorbance was measured at 540 nm on spectrophotometer. All the tests were performed in triplicates.

2.4.4 Biodegradation Percentage Determination Assay

Tannin concentrations in the supplemented media were also measured by bovine serum albumin (BSA) precipitation assay during time intervals [27]. For biodegradation experiments, in the media supplemented with upto 50 g/L of tannic acid, tannin concentration was measured by bovine serum albumin (BSA) precipitation assay after 24, 48, 72, 96, 120 and 144 hours of incubation and the biodegradation percentage was calculated [28]. 5 mL of the bacterial suspensions or the cell free broth was

centrifuged (3,000 rpm at 4 °C for 10 min) and the supernatant was taken to determine tannins. The pellet obtained was air dried and weighed to check the increasing biomass of the bacterial sample. For each sample, 1 mL of a 1 mg BSA/mL acetate buffer stock solution was combined with 1 mL of tannic acid solution and precipitation reactions were allowed to proceed under refrigeration for 18 hr after which samples were centrifuged (3,000 g for 10 min) and the precipitate was dissolved in SDS solution (1% w/v). The aliquot of 1 mL of resulted solution was combined with 3 mL TEA- SDS solution (7%TEA & 1% SDS). After several minutes 1 mL of FeCl_3 was added and the optical density was measured after 60 min incubation at room temperature, then absorbance was measured at 520 nm, zeroing the spectrophotometer with a tube containing all of the reagents plus water in place of the extract. Tannin contents were determined according to calibration curve ($R^2=0.971$) which was constructed from commercial tannic acid ranging from 10- 50 g/L.

2.4.5 Molecular Identification

Bacterial DNA was extracted from the samples, the final DNA concentration determined and DNA quality was checked by 1% AGE after purification. The PCR product was then sequenced using the primers and the unincorporated PCR primers and dNTPs from PCR products were removed. Single-pass sequencing was then performed on each template using below 16s rRNA universal primers and the fluorescent-labeled fragments were purified from the unincorporated terminators. The samples were resuspended in distilled water and subjected to electrophoresis. RNA sequence analysis was employed to characterize and identify bacterial species. The 16S ribosomal RNA sequences obtained were analyzed by using the BioEdit software and then aligned by ClustalW. The 16S rDNA sequence was compared with the sequences in GenBank by using standard BLASTn search to check for similarities with the already found strains of the bacteria *Bacillus subtilis*.

3. Results and Discussion

3.1 Identification and Characterization of the Isolated Bacteria

The research study was initiated to isolate, cultivate and identify tannic acid degrading bacteria from the decaying portion of barks. Decaying barks were chosen as a probable source for the isolation of tannin degrading bacteria as it had high probability of having bacterial degraders that would have breached the guard of the tree's first defence against microbes, tannins. It was thought that decaying portions of the bark will be the apt source to isolate tannin-degrading bacteria, where tannins are no longer able to inactivate the invasive extracellular enzymes of microbes and where microbes that can invade the protective layer of tannins could possibly be present.

Bark samples were inoculated on minimum salts medium to isolate tannic acid degrading microorganisms. Since tannic acid can form complexes with proteins and other compounds, it is difficult to use organic sources of carbon, nitrogen and proteins in the growth medium [29]. Therefore, isolation of tannase producers required a specific media, such as, MSM with tannic acid as the source of carbon [30]. Their presence was confirmed by growing them in increasing concentration of tannic acid (0.1-0.5%). Three isolates (I-1, I-2, I-3) showed growth on medium containing 0.1-0.5% tannic acid. These three isolates were further cultivated for colony, physiological and biochemical characterization as shown in Table 1 and 2.

Table 1 Morphological characteristics of the three isolates

	Gram stain	Morphology	Colony shape	Colony size	Margin
Isolate 1	Positive	Rod shaped	Irregular white	<1mm	Entire
Isolate 2	Positive	Rod shaped	Circular white	<1mm	Entire
Isolate 3	Positive	Cocci	White rhizoid	>1mm	Filiform

Table 2 Biochemical characteristics of the three isolates

Biochemical Test	Isolate 1	Isolate 2	Isolate 3
Methyl Red	Positive	Positive	Positive
Voges Proskauer	Negative	Negative	Negative
Indole Production	Negative	Negative	Negative

These results of the biochemical tests that were performed, indicate that all the isolates could ferment glucose but could not produce the enzyme tryptophanase or butane-2,3-diol.

3.2 Tannase Activity

3.2.1 Maximum Tolerable Concentration

The isolate I-1 showed a maximum tolerable concentration of tannins of 50 g/L among the three isolated bacteria and was thus chosen as the most efficient tannin degrading bacteria for further tests.

3.2.2 Visual Detection Assay

Tannin-protein complex (T-PC) degrading activity of the isolate-1 was checked on plate containing nutrient agar coated with tannic acid, forming T-PC, which shows an opaque shiny color of the medium. A zone of clearance formed by reduction of opacity around the colonies of I-1 was taken as indication of the hydrolysis of T-PC. Acidic pH around the colonies, tested by touching pH indicator strip near the colonies was indicative of the release of tannic acid. The clearing zone turned brown after 24 hr, presumably because of the oxidation of tannic acid released after the hydrolysis of T-PC as seen in Fig. 1 [31].

Fig. 1 Tannase activity test

3.2.3 BSA Precipitation Assay

Tannin concentrations were also measured by bovine serum albumin (BSA) precipitation assay and the biodegradation percentage was calculated. One of the potential mechanisms of polyphenols action on bacterial cells is that polyphenols can bind to bacterial cell membranes in a dose- dependent mode [32]. In biodegradation experiment, tannic acid utilization as the sole carbon and energy source resulted in visual turbidity increase with a simultaneous production of acidic metabolites as indicated by an additional decrease in the pH of the medium [24]. The primary pH of culture fluid was 5.9 that decreased to 3.39 – 4.15 after 96 hrs of incubation. A gradual decrease in the concentrations of tannic acid was observed with the increasing incubation period, noted up to 144 hours as seen in Fig. 2. Tannic acid degradation was at its peak after 72 hrs of incubation. Also, the degradation percentage of tannic acid at concentrations of 10 g/L, 20 g/L, and 30 g/L reached more than 90% after 144 hrs incubation, while for concentrations of 40 g/L and 50 g/L, the degradation percentage reached a maximum of up to 60%.

Fig. 2 Graph indicating tannic acid degradation with increasing time

3.2.4 Tannase Specific Assay

Tannase specific assay was also done using the Miller's method where tannase breaks tannic acid which acts as a substrate, forming Gallic acid and liberating glucose. Tannase activity was determined by estimating the reduced glucose liberated using 3,5-dinitrosalicylic acid reagent. From the results below (as shown in Fig. 3) it was clear that the enzyme activity of isolate giving 51.61 U/mL was the optimum after 96 hours of incubation <https://doi.org/10.30799/jnpr.100.21070201>

for enzyme production. The graph below represents the enzyme activity of the isolate at different hours. The method determines the amount of glucose liberated in the production medium and so it gives such high values for enzyme activity.

Fig. 3 Tannase activity of the *Bacillus subtilis* strain

3.2.5 Molecular Identification

The isolated bacterial strain was subjected to 16S rRNA sequencing and was compared with the available 16S ribosomal sequences in the NCBI database using BLASTn. The tannin degrading bacteria has been enrolled into a cluster containing *Bacillus* sp. and was found to have 96% identity with *Bacillus subtilis* as shown in the Fig. 4. While the multiple sequence alignment and phylogenetic tree construction reveals that the *Bacillus subtilis* strain used in this study is closely related to *Bacillus methylophilus* strain MER_163 as well as to *Bacillus subtilis* strain 10A but is not one among the already found strains. The newly identified strain of *Bacillus subtilis*, namely I-1, was then submitted to NCBI under the accession number MH330408.

Fig. 4 Phylogenetic tree showing the difference from the already found strains

4. Conclusion

In the degradation mechanism of tannin, microbes play an important role by secreting the enzyme tannin acyl hydrolase or tannase, which is capable of catalyzing gallotannins to gallic acid and glucose. And although gallic acid is widely distributed in nature, it is difficult for bacteria to use it as a carbon source for growth at neutral or alkaline pH, unlike glucose. In this study, tannic acid degrading *Bacillus* sp was isolated from decaying tree bark. Bacterial strains degrading tannic acid have been previously isolated from different sources such as oil mill wastes, goat, cow or mouse feces pistachio soft hulls and soil. Our findings along with the fact this strain is able to grow on higher concentrations of tannic acid (that is up to 50 g/L) give them an amazing potential for its application in bioremediation, waste water treatment, and also reduction of antinutritional effects of tannins in animal feeds. It could also be very valuable in industry for production of tannase. Furthermore, the ability of the isolate (*Bacillus subtilis* strain I1) characterized in this study provides novel insights for the role it can play in composting of tannin containing wastes. The research on biodegradation of tannins is in an incipient stage and further studies must be carried out. Such studies apart from helping us to understand the phenomenon of nature, ecology of microbial succession and allelopathic effects, will also aid to exploit the potential of various tannase for large scale production and its applications in food, fodder, and medicine and tannery effluent treatment. This bacterial isolate may have use in clarification of fruit juices, residues of coffee pulp, thus being a good alternative to solve the big problem that represents the accumulations of this agro industrial byproduct in some developing countries like Brazil, Mexico, or Colombia. Furthermore, these tannase producing bacteria may find application in food (clarification of fruit juices), pharmaceutical industries and can be used in the study of genes involved in the production of tannase, an industrially important enzyme.

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