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Research Paper

Isolation of tannase producers from soil

Vora Dipak, *Sarang Sheela
Department of Microbiology, Ramnarain Ruia College, Mumbai, Maharashtra, INDIA

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Abstract

The enzyme tannase hydrolyses the ester bonds of tannic acid to release gallic acid and glucose. Tannic acid is known to be bacteriostatic but a few organisms are resistant to it by producing tannase enzyme. Frequently used assay for tannase is the isolation of tannase producers in the presence of tannic acid on a nutrient plate. An attempt has been made to find isolation procedures for tannase producers and eliminate the false positive results. Tannic acid is an integral and important plant constituent. Thus soil samples would logically be an excellent source for the isolation of tannase producing bacteria. These samples were collected from Mumbai and Thane region. The samples were plated onto nutrient agar plates containing 2% tannic acid. Isolates showing a halo of clearance around the colonies were selected and screened to confirm their tannase producing ability. Amongst the cultures obtained, one bacterial tannase producer was obtained which showed relatively high enzyme activity. The bacterial isolate was identified and characterised using various biochemical tests. This paper highlights the use of different methods for the isolation of tannase producers, thus, eliminating chances of selecting an isolate that demonstrates false positive results.

Keywords: Identification, Paper Chromatography, Soil, Swarm Plate Assay, Tannase, Visual Assay, elimination of false positive results.

Introduction

Tannins are reported as the fourth most abundant group of compounds found in plants. They are water soluble polyphenols with a molecular mass ranging from 0.3 to 5 k Da [1] and are known to be inhibitory against various organisms. Their role appears to be to protect the vulnerable parts of plants from microbial attack by inactivating the invasive microbial extracellular enzymes [2]. Tannins and other polyphenols are known to form irreversible complexes with proteins. However, many fungi, bacteria and yeasts exhibit resistance to tannins and can thus proliferate in their presence. Fungi such as *Aspergillus*[3,4], Penicillium[5], Trichoderma[6] and bacteria such as *Bacillus* [7,8], *Lactobacillus* [9,10], *Klebsiella*[11] demonstrate strong tannase producing abilities. Due to their ability to form complexes with proteins and a number of other compounds, it is difficult to use organic sources of carbon, nitrogen and proteins in the growth medium [12]. Hence, isolation of tannase producers would require specific media with tannic acid as the source of carbon.

Tannase (tannin acyl hydrolase, E.C. 3.1.1.20) is an inducible enzyme. It hydrolyses ester and depside linkages in hydrolysable tannins (such as tannic acid), liberating glucose and gallic acid (Figure 1).

Tannase has exhibited important applications in the food, beverage, chemical and pharmaceutical industry. Tannase is used commercially in the production of high grade leather, clarified beer and fruit juice, coffee flavoured soft drinks, premium tea, cosmetics etc [13]. It is also used to produce gallic acid, which in turn is an important intermediate in the manufacture of Trimethoprim (anti malarial drug) and the antioxidant propyl gallate.

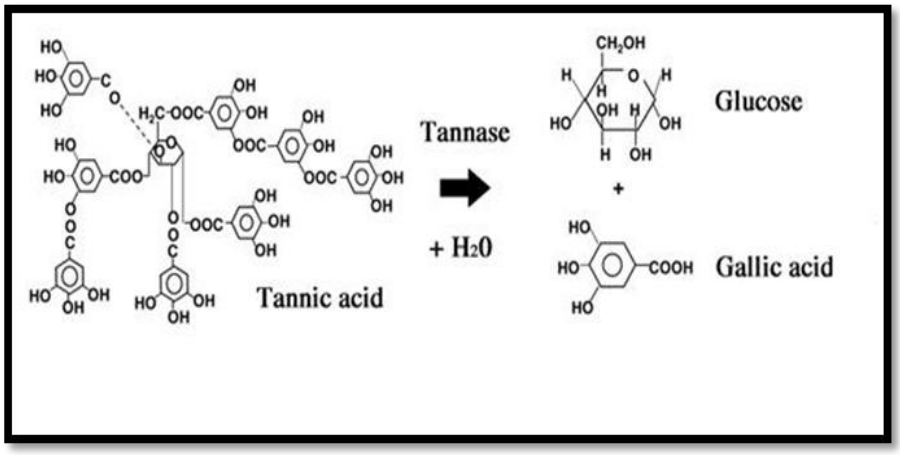

Figure 1: Tannase acting on tannic acid to release gallic acid and glucose

Conventionally gallic acid is produced by the chemical hydrolysis of tannic acid. Disadvantages of this technique are the resulting high cost, low yield and low purity of gallic acid. Considering that the global requirement for gallic acid is approximately 8000 tons/year[14], production of gallic acid biologically is a very important application. Biologic production of gallic acid increases the economic efficiency of the procedure as well as allows for better purity. From an environmental perspective, tannases have been known to degrade tannery wastes which are otherwise difficult to clarify. Besides this, a recent study on tannin degrading micro-organisms concluded that the tannase producing bacteria increase in number in the gut of patients suffering from colorectal cancer. The growth of these bacteria in the patients is considered to be related to the prevention of the tumour progression [15].

The assay usually adopted for detecting tannase producers is to isolate the bacterial culture on nutrient agar/ minimal media plates containing 2% tannic acid. A positive result is indicated by the appearance of a zone of clearance around the colonies as the tannic acid around the colonies is utilized by the organism. However, a recent study has shown that sometimes a halo may also be produced due to an alkaline shift in the medium during the bacterial growth causing the dissociation of the tannin protein complex in the medium [16]. Hence, this test was used as a preliminary test and the positive isolates were further tested for their ability to produce tannase. A bacterial tannase producer was obtained which showed a relative strong tannase activity.

Materials and Methods

All materials used are from Merck and Himedia, India unless stated otherwise.

Sampling and Preliminary analysis

Twenty soil samples were collected from different areas in the Mumbai (18.9750°N, 72.8258°E) and Thane (19.1724°N, 72.9570°E) districts. Samples were enriched in nutrient broth containing 0.1% tannic acid for 24 hours at ambient temperature under stationary conditions. Each sample was then streaked onto the 3 nutrient agar plates containing 2% tannic acid.

The isolates so obtained from the preliminary assay were grown on nutrient agar slants and preserved at 8-10°C in the refrigerator. The cultures were regularly passed on to fresh slants at 2 week intervals. They were subjected to the following tests. 24 hrs old culture growth from nutrient broth were used for these tests.

Swarm plate assay [17]

Minimal microbial medium was used for the assay. The composition of 5X minimal media was Disodium Phosphate (Anhydrous) (33.9 g/l), Monopotassium Phosphate (15.0 g/l), Sodium Chloride (2.5 g/l), and Ammonium Chloride (5.0 g/l). The media was diluted to 1X concentration with distilled

water. Magnesium sulfate and Calcium Chloride were added so as to achieve a final concentration of 0.2% and 0.01% respectively in the 1X medium.

The test was carried out using Minimal media plates containing tannic acid (1%) as sole source of carbon, tetrazolium chloride (0.05%) and 0.2% of agar. The above isolates were spotted and were incubated for 24-48 hrs at ambient temperature.

Visual Detection assay

Visual assay was performed as per the protocol of Osawa [18]. Substrate media contained 33mM Monosodium phosphate and 20 mM Methyl gallate in distilled water. A dense suspension (0.5 O.D.) of the organisms been tested was prepared in sterile saline and 0.1 ml of this suspension was inoculated into tubes containing 5 ml substrate medium which was incubated for 24 hours at 37^0C. This was followed by centrifugation at 10000 rpm at 4 ^{0}C and the pellet discarded. Supernatant obtained was mixed with equal amount of saturated sodium bicarbonate (pH 8.6) and incubated for 1 hour in dark under ambient conditions. Development of green or brown colour is considered a positive test.

Detection of degradation products

Paper chromatography was performed according to method of Katwa[19]. Test samples and appropriate standards were spotted onto Whattman No. 1. The spots were allowed to air dry. The solvent system was 6% acetic acid in water. The paper was then dried and final products were detected using 1% Ferric chloride in 30% methanol.

Results and Discussion

Sampling and Preliminary analysis

Tannic acid is a plant constituent. It is usually found in the tree barks. It therefore can be derived that the tannase producing bacteria could most probably be found in soil under them. Of the twenty soil samples, seventy isolates demonstrated a halo around the colonies (Figure 2). These seventy cultures were screened further for tannase activity.

Figure 2: Halo around the colony on nutrient agar plate

Swarm Plate Assay

Bacteria inoculated into the centre of a nutrient-rich plate fortified with less than 0.3% agar will consume nutrients locally; generate a nutrient gradient and chemotax up the gradient through the pores in the agar. In this assay, Tannic acid is added to medium as the main source of carbon and the culture is added in the wells. Organisms which show movement and growth in a semi solid media containing tannic acid are the ones which have high tolerance for the compound (Figure 3).

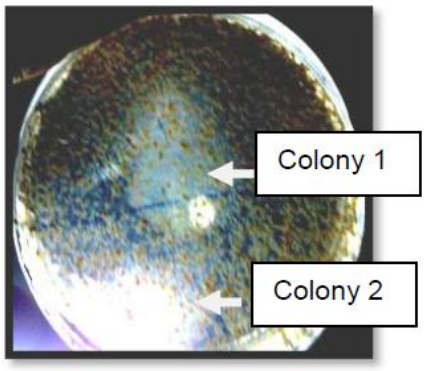

Figure 3: Two colonies were spot inoculated onto the agar plate and showed positive result

Visual detection assay

The substrate medium was made alkaline by the addition of sodium hydrogen carbonate to facilitate the non enzymatic conversion of gallic acid to polymers of o-quinone derivatives resulting in a green to brown color. Thus, organisms demonstrating tannase activity alter the colour of the supernatant to show a green to brown coloration while the ones with no activity show an orange/yellow coloration. (Figure 4)

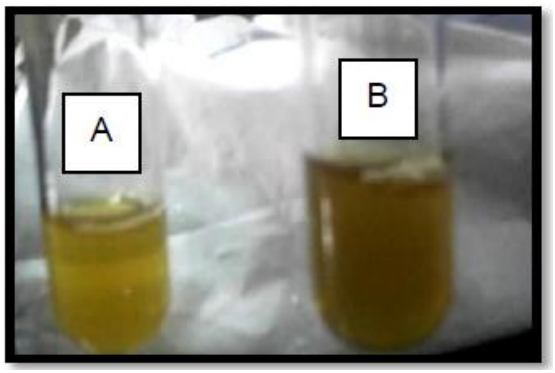

Figure 4: Figure shows visual assay test (A: negative control; B: test)

Paper Chromatography

Tannase breaks down the tannic acid to gallic acid. Paper chromatography was performed with standards tannic acid and gallic acid. Samples were also spotted on the same paper. Samples were obtained from nutrient media containing tannic acid. Culture supernatant was used as the samples (Figure 5).

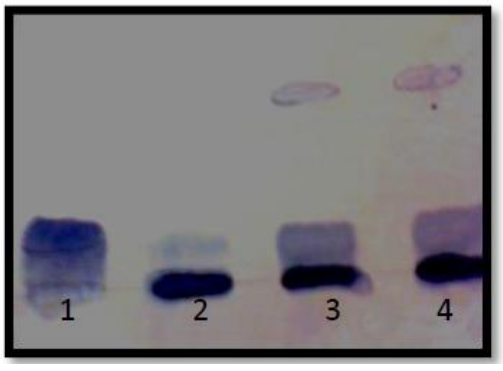

Figure 5: Paper Chromatography

Lane 1: Gallic acid, Lane 2: Tannic acid, Lane 3, 4: Samples

Identification of Tannase producers

One tannase producer was obtained which showed the positive results in all the above tests. This was isolated and enriched in nutrient broth. It showed red pigment when incubated at 30^0C but no pigment when incubated at 37^0C. The culture showed pinpoint colonies on nutrient agar. Identification tests were conducted according to the Bergey's manual. The organism is identified as *Serrratia marcesans* (Table 1).

Table 1: Identification tests for soil isolate

Tests	Observation and Conclusion
Gram staining	Gram negative Coccobacilli
Carbohydrate fermentation: • Lactose • Glucose • sucrose	 • no acid or gas • acid and gas • acid only
Triple sugar slant	Yellow butt. Glucose is fermented. Hydrogen sulfide is not produced
Oxidase	Positive
Catalase	Positive
Growth in anaerobic medium	Growth seen. Facultative anaerobe
Citrate	Growth seen and slant turned blue. Positive
Casein hydrolysis	Zone of hydrolysis seen. Positive
Salt tolerance	Salt tolerance is 7%
Mr	Turned red. Negative
Vp	Red ring. Positive
Nitrate	Red coloration after addition of sulfanilic acid. Positive
Motility	Motile.
Gelatin hydrolysis	Zone of hydrolysis seen. Positive
XLD	Growth seen
Starch hydrolysis	No clearing after addition of iodine. Negative
Indole	No pink ring. Negative
Dnase	Purple hydrolysis zone observed. Positive
Blood haemolysis	Zone of haemolysis seen with green discoloration. Positive

Conclusion

This paper discusses various methods for isolating strong bacterial tannase producers efficiently. Conventionally, tannase producers are detected on tannic acid containing nutrient agar plates and observing for a zone of clearance around the growth. This test could lead to false positive results due to the secretion of alkaline products by the bacteria into the media. Presence of an alkaline pH also generates a clearance. Hence, it is advisable to use the above test solely as a preliminary test and confirm with the help of additional tests.

The use of natural tannins has also been suggested for the isolation of tannase producers. The reason for this is that the bacteriostatic activity of tannic acid could be offset by the presence of other components in these natural tannins. Thus the use of natural tannins could probably enhance our ability to detect tannase producers more effectively. However, though this method accounts for the toxicity of tannic acid it suffers from the disadvantage in that prior treatment of the substrate by lyophilisation is required before its incorporation into the media and the problem of false positive results has not been addressed at all.

This paper discusses a more evolved but effective set of techniques that could be used to detect and isolate tannase producers. Further, we can effectively detect those isolates which possess relatively stronger activity and can eliminate false positive results. Minimal media which uses tannic acid as a sole source of carbon clearly demonstrates the ability of the isolate to utilise tannic acid. The use of visual detection assay detects the presence of the activity and also demonstrates the strength of the

activity which can be assessed based on the intensity of colour generated. Finally, paper chromatography method separated and helps in indentifying the products of hydrolysis. Thus, the isolation techniques mentioned in this paper may be used effectively with no previous treatment to the substrate and the series of three tests ensures the accuracy of the observations.

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