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Production of Novel Cell-Associated Tannase from Newly Isolated *Serratia ficaria* DTC

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Five strains of tannic acid degrading bacteria were isolated and identified by phenotypic characterization. All the five isolates showed cell-associated activity, whereas only three showed extracellular activity. *Serratia ficaria* DTC, showing the highest cell-associated activity (0.29 U/l), was selected for further shake-flask studies. Tannase synthesis was growth associated and reached the peak in the late stationary phase of growth. Organic nitrogen sources enhanced the tannase production. Peak tannase production of 0.56 U/l was recorded in the medium having the initial pH of 6. The pH and temperature optima of the enzyme were found to be 8.9 and 35°C, respectively. This is the first report of cell-associated activity in the case of bacterial tannase. Cell-associated tannase of *Serratia ficaria* DTC could be industrially important from the perspective of its activity at broad temperature and pH ranges, and its unusually high activity at pH 8.9.

Keywords: Cell-associated tannase, extracellular tannase, *Serratia ficaria*

Tannins are the fourth most abundant plant constituent after cellulose, hemicellulose, and lignin. Tannin protects the vulnerable parts of plants from microbial attack by inactivating the invasive microbial extracellular enzymes. However, many bacteria have developed the ability to grow in the presence of tannins. The mechanisms by which bacteria can overcome inhibition include tannin modification, degradation, dissociation of tannin–substrate complexes, tannin inactivation by high-affinity binders, membrane modification, and metal ion sequestration [22]. Tannin acyl hydrolase (E.C. 3.1.1.20), commonly known as "tannase," catalyzes the hydrolysis of ester and depside bonds in hydrolyzable tannins [10]. Many bacteria have the ability to produce tannase, which reduces the concentration of harmful tannin and also produces the monomers, some of which in turn are used as carbon and energy sources [6].

Major applications of this enzyme are in the production of gallic acid, instantaneous tea, and acorn wine. Gallic acid is used for the manufacture of an antimalarial drug, trimethoprim, and in the synthesis of esters, such as propyl gallate, used as antioxidant in the food industry. The enzyme is also used as a clarifying agent in some wines, juices of fruits, and refreshing drinks with coffee flavor. The enzyme has potential uses in the treatment of tannery effluents and pretreatment of tannin-containing animal feed [7].

Several reports of production of bacterial tannase are available. Productions of extracellular tannase from genera *Bacillus*, *Staphylococcus*, and *Klebsiella* [7], and species *Bacillus licheniformis* [15], *Bacillus cereus* [16], and *Lactobacillus plantarum* [2] are noteworthy. There are quite a few reports of cell-bound and intracellular tannase activities in molds [13,20]. Several studies related to cell-associated enzymes of bacteria have been carried out [1, 8, 11, 17, 19, 21]. However, there are no reports of cell-associated tannase activity in bacteria.

In this communication, we report the production of cell-associated tannase by newly isolated bacterial strains. This includes the isolation and identification of the organisms, shake-flask studies, and properties of the cell-associated enzyme.

Materials and Methods

Isolation and Identification of Tannase-Producing Bacteria

Samples were collected from the effluent pit of a tea processing factory and from rotting skin of grapes into fresh glass vials. About 1 g of sample was added to 100 ml of nutrient broth, and incubated in an incubator shaker for 48 h at 30°C, 150 rpm. One ml of turbid broth was inoculated into selection medium having the following composition (g/l): tannic acid, 1.25; $(NH_4)_2SO_4$, 1.3; yeast extract, 1.0; KH_2PO_4, 0.37; $MgSO_4{\cdot}7H_2O$, 0.25; $CaCl_2{\cdot}2H_2O$, 0.07; $FeCl_3$,

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0.02. One hundred ppm of Cycloheximide was used to suppress the possible fungal growth. The pH was adjusted to 4.8. Tannic acid was filter sterilized and added after the sterilization of other media ingredients. The cultures showing substantial growth were transferred to fresh medium. After three more transfers, the turbid broth was diluted appropriately and spread plated on nutrient agar and discrete colonies were picked up and preserved at 4°C on nutrient agar slants. Strains were identified based on their morphological, physiological, and biochemical characteristics according to *Bergey's Manual of Determinative Bacteriology* [4].

Growth Studies

Seed culture was prepared by growing the organism in 50 ml of sterile medium in a 100-ml conical flask for 24 h in an incubator shaker at 30°C, 150 rpm. Batch fermentation was carried out in 250-ml Erlenmeyer flasks containing 100 ml of fermentation medium inoculated with 2% (v/v) seed culture. The fermentation medium contained (g/l) tannic acid (Sigma), 5.0; $MgSO_4$, 0.5; NH_4NO_3, 3.0; KH_2PO_4, 0.5; K_2HPO_4, 0.5; mineral salt solution, 10. Mineral salt solution had the following composition (g/l): $(NH_4)_6MoO_{24}{\cdot}4H_2O$, 0.1; $MnCl_2$, 2.0; $CuSO_4{\cdot}5H_2O$, 0.2; $CoSO_4$, 2.8. Steam-sterilized mineral salt medium and filter-sterilized tannic acid were added aseptically to the basal medium after sterilization. The pH was adjusted to 5.5. The fermentation was carried out at 30°C in a rotary shaker at 150 rpm. Sediments after centrifugation were dried at 95°C for 36 h to assess dry cell weight. The broth was centrifuged (5,500 ×*g*, 15 min), and the supernatant was assayed for extracellular activity, and the sediment was assayed for cell-associated activity.

Tannase Activity

Tannase activity was estimated by reaction with tannic acid as substrate followed by the reaction with methanolic rhodanine, as described elsewhere [23]. The quantity of gallic acid released during hydrolysis of tannic acid represents the tannase activity. The sensitivity of the assay was 36×10^{-6} U/ml. The culture broth was centrifuged at 5,500 ×*g* for 20 min, and then the cell pellet was washed and resuspended in 0.1 mol/l acetate buffer (pH 5.0) and used to measure cell-associated tannase activity. The dry cell weight per ml of cell suspension determined was used to calculate the cell-associated tannase activity and expressed as U/l. Cell-free supernatant was used to measure extracellular tannase activity. One unit of enzyme activity was defined as 1 μmole of gallic acid released per minute in the assay conditions. All the determinations were performed in duplicate and the results are given as the mean values.

Effect of Organic Nitrogen Sources

The effect of medium having inorganic and organic nitrogen sources (M_1, M_2, and M_3) on cell-associated tannase production was tested. The composition of the medium along with other ingredients as mentioned earlier was as follows:

M_1, Only ammonium nitrate (3 g/l).

M_2, Ammonium nitrate (3 g/l)+Casein hydrolysate (3 g/l) (Fluka).

M_3, Ammonium nitrate (3 g/l)+Casein hydrolysate (3 g/l)+Yeast extract (10 g/l) (Himedia India).

Optimum Medium pH

The fermentation medium supplemented with 3 g/l of casein hydrolysate and 10 g/l of yeast extract was taken, and the initial pH was adjusted to 3, 4, 5, 6, and 7 using 0.1 M NaOH and 0.1 M HCl.

Effects of Temperature and pH

The effect of temperature on cell-associated tannase activity was tested at 10−80°C. The pH profile was measured at 30°C at various pHs of 0.1 M buffers: 2.5−3.5 using Glycine-HCl buffer; 4.0−6.0 using citrate buffer; 6.5−7.5 using phosphate buffer; 7.6−8.6 using Tris-HCl buffer; 8.7-10.0 using Glycine-NaOH buffer.

Results and Discussion

Isolation and Identification

Five pure cultures were isolated and named as DTC, DT1, DTP, DT5, and DT6. These isolates were identified based on their colony morphology; Gram's reaction; presence of spores; motility; ability to grow at various temperatures, pHs, and NaCl concentrations; ability to show anaerobic growth; and various biochemical tests. All the five strains were deposited and the identification has also been confirmed from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India as *Serratia ficaria* (MTCC 8930), *Serratia marcescens* (MTCC 8927), *Serratia marcescens* (MTCC 8931), *Microbacterium terregens* (MTCC 8928), and *Providencia rettgeri* (MTCC 8929).

Rapid growth was observed in the production medium during shake-flask trials, indicating the bacteria are using tannic acid as the source of carbon and energy. Utilization of tannic acid requires the degradation and liberation of products such as gallic acid, glucose, *etc.*, through enzymatic hydrolysis. Some of these cultures showed no sign of extracellular tannase activity, and therefore we measured the tannase activity of the cell suspension. All the five isolates showed tannase activity in cell suspension, suggesting the ubiquitous nature of this phenomenon in tannin-degrading bacteria. This is the first report of cell-associated tannase activity in bacteria, although cell-associated enzyme is not uncommon in bacteria [8, 11, 17, 21].

Growth Studies

The samples were withdrawn every 4 h to estimate the cell-associated tannase activity, extracellular tannase activity, and dry cell weight. All the five isolates showed cell-

Table 1. Cell-associated and extracellular tannase activities of five isolates.

Isolate	Name	Cell-associated activity (U/l)	Extracellular activity (U/l)
DT1	*Serratia marcescens*	0.15	0.28
DTC	*Serratia ficaria*	0.29	0.31
DT5	*Microbacterium terregens*	0.026	-
DT6	*Providencia rettgeri*	0.14	1.2
DTP	*Serratia marcescens*	0.017	-

associated tannase activity, whereas DT5 and DTP failed to show extracellular activity (Table 1). Among the five isolates, *Serratia ficaria* DTC showed the highest cell-associated tannase activity of 0.29 U/l among all the isolates, which was taken for further trials. Growth studies revealed that the peak activity of 0.29 U/l was recorded after 40 h of incubation, which corresponds to the late stationary phase of growth (Fig. 1). Growth studies in shake flasks of the selected isolate *Ser. ficaria* indicated that the production of cell-associated tannase is growth associated and reaches the peak in the late stationary phase, contrary to many reports of peak production in the exponential phase of growth [8, 12, 19] in the case of extracellular tannase. However, Mondal *et al.* [16] reported that the peak extracellular tannase production occurred at the stationary phase in *B. cereus*.

Effect of Organic Nitrogen Sources

M_3 and M_2 media showed 3.8 and 2.9 times higher enzyme activity than M_1 medium, indicating that cell-associated tannase production is favored by organic nitrogen sources in the medium (Fig. 2 and 3). Peak enzyme production was observed after 24 h of incubation in the M2 and M3 media, whereas it took 40 h to reach peak enzyme production in M1 medium. The tannase activities per gram of biomass for the M1, M2, and M3 media were found to be 0.73 U/g, 1.33 U/g, and 1.21 U/g, respectively. This clearly shows that organic nitrogen sources increased the enzyme yield per liter because of increased biomass. However, the enzyme yield per gram of biomass was more in M2 medium compared with M3 medium, which suggests that a mere increase in biomass does not guarantee higher tannase yield. Moreover, the concentration of tannic acid, which acts as an inducer, remained the same in all the media. Several authors have reported media optimization for cell-bound enzymes [1, 24]. However, no study has been conducted on the relation between the increase in cell mass and enzyme activity per gram of cell mass. Perhaps a complex relationship exists between them, which requires further investigation.

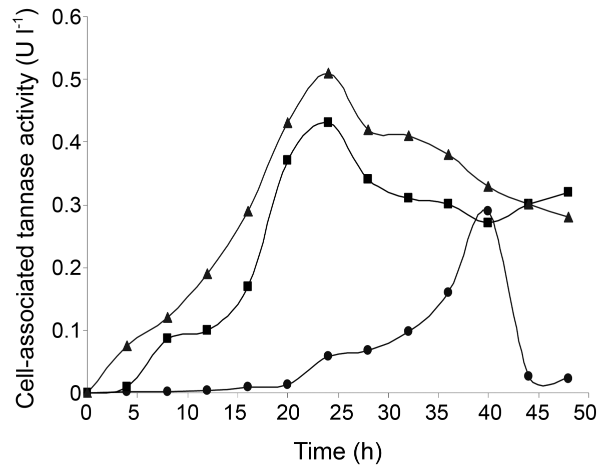

Fig. 2. Cell-associated tannase synthesis in M_1 medium (●), M_2 medium (■), and M_3 medium (▲).
M_1, Ammonium nitrate (3 g/l); M_2, Ammonium nitrate (3 g/l)+Casein hydrolysate (3 g/l); M_3, Ammonium nitrate (3 g/l)+Casein hydrolysate (3 g/l)+Yeast extract (10 g/l).

Optimum Medium pH

Significant enzyme production was observed in the pH range of 5 to 7.5. Maximum enzyme production was observed in the medium having the initial pH of 6.0 (Fig. 4). This could be explained by the fact that this bacterium was isolated from rotting vegetative matter, which was slightly acidic in nature.

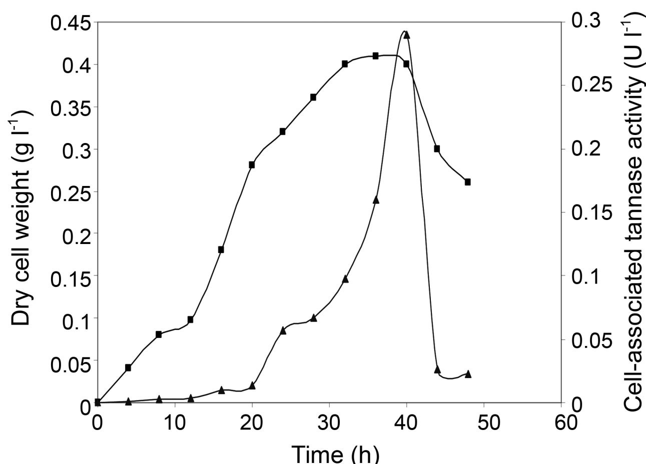

Fig. 1. Time course of biomass growth (■) and cell-associated tannase activity (▲) of *Serratia ficaria* DTC.

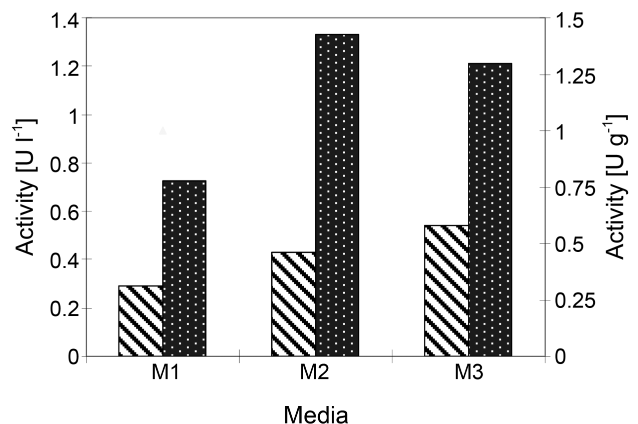

Fig. 3. Comparison of tannase activity per liter ▧ and tannase activity per gram of biomass ▦ for media M1, M2, and M3.
M_1, Ammonium nitrate (3 g/l); M_2, Ammonium nitrate (3 g/l)+Casein hydrolysate (3 g/l); M_3, Ammonium nitrate (3 g/l)+Casein hydrolysate (3 g/l)+Yeast extract (10 g/l).

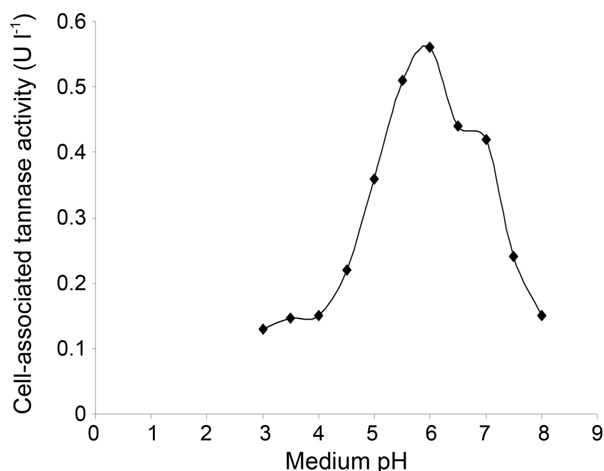

Fig. 4. Effect of initial pH of the medium on cell-associated tannase synthesis in *Serratia ficaria* DTC.

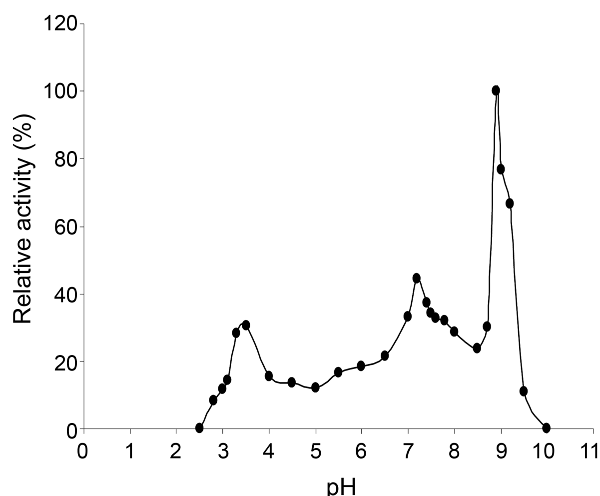

Fig. 6. Effect of pH on cell-associated tannase activity of *Serratia ficaria* DTC.
The relative activity of 100% is 7.69 units of enzyme per liter.

Effects of Temperature and pH

It has been observed that cell-associated tannase enzyme was active over the temperature range of 10 to 90°C and showed the maximum activity at 35°C (Fig. 5). Cell-associated tannase showed good activity in the range of 30−45°C, and maintained about 20% of its peak activity in the range of 45−80°C. Similar findings were reported by Sinsuwan *et al*. [21] in the case of cell-bound proteinase from *Virgibacillus*.

The cell-associated enzyme was active in the pH range of 2.8 to 9.5 and showed three significant peaks at 3.5, 7.2, and 8.9. The highest activity was recorded at pH 8.9 (Fig. 6). It showed activity in pH 2.8−9.5, which is quite remarkable for an enzyme produced by an organism growing at pH 3.0−7.5. Kopecny and Wallace [11] reported similar findings in cell-bound protease from rumen bacteria.

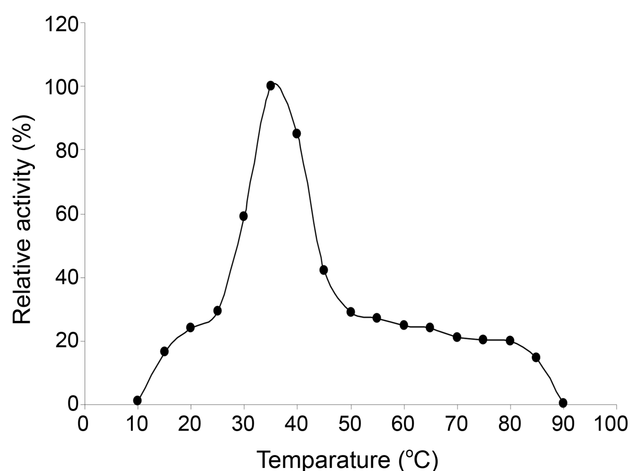

Fig. 5. Effect of temperature on cell-associated tannase activity of *Serratia ficaria* DTC.
The relative activity of 100% is 2.0 units of enzyme per liter.

Typically, the pH optimum for bacterial tannase is about 4.5−5.8 [7, 14−16] and for fungal tannase is 5.0−6.0 [3, 13, 18]. Contrary to this, the cell-associated tannase showed 30.5%, 44.5%, and 100% of its peak activity at pH 3.5, 7.2, and 8.9 respectively. Probably, this is the first report of any tannase showing peak activity beyond the 4.5−6.0 pH range. The existence of three peaks also implied that the cell-bound tannase might contain several tannases [11].

It is a well-known fact that treatment of biomass with alkali at pH 8−12 for 20−30 min results in lysis of cells. The extent of lysis varies from one organism to another [9]. The increase in tannase activity could be due to lysis of cells with concomitant release of intracellular tannase enzyme or a change in permeability of the membranes, which lead to higher access of substrate molecules to membrane-bound tannase. Nevertheless, the extreme alkaline pH renders most of the released enzyme useless owing to inactivation in most of the cases. However, despite of the alkaline pH (8.9), tannase showed 2.24 times that of activity at pH 7.2, which requires further investigation.

Cell-associated tannase of *Serratia ficaria* DTC could be industrially important from the perspective of its activity at broad temperature and pH ranges, especially its unusually high activity at pH 8.9. This could be an ideal candidate for several industrial applications, including tannery effluent treatment.

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