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Purification, Characterization and Application Study of Bacterial Tannase for Optimization of Gallic acid Synthesis from Fruit Waste

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Tannase produced extracellularly by the bacterial strain *Bacillus haynesii* SSRY4 MN031245 was purified in step-wise manner through ammonium sulphate precipitation, dialysis, followed by anion exchange chromatography. Tannase was purified to 42.0-fold with 36.30% enzyme yield. The enzyme was relatively stable from 30 to 50°C and pH (4.0–6.0) for up to 4 hours. Partially purified tannase (16.80 U/ml) was able to synthesize 20.304 mg/ml gallic acid from the fruit waste under optimized conditions. The results of application study suggest that bacterial tannase could provide a new source for Gallic acid synthesis from the fruit waste for industrial applications. Our research findings could provide a value chain to fruit waste and help in reducing the waste generation from fruit processing industries.

Keywords: *Bacillus haynesii*, Enzyme, Hydrolysis, Quantification, Yield

Introduction

Enzymes play a significant role in vast range of industrial processes and applications. Tannase is an inducible hydrolase that can be efficiently employed for the hydrolysis of hydrolysable tannins.[1] Even though fungal tannase has been widely studied and utilized for industrial applications; the application of fungal isolates for the large-scale production of tannase is limited owing its genetic complexity and slower growth rate.[2] A vast majority of tannase of fungal origin consists of more than one subunit.[3] This renders it less suitable for over expression, purification and in applications requiring low molecular weight enzymes compared to bacterial tannase.[4] Purification of tannase has been attempted earlier using one or the other purification protocols. Various techniques viz high-performance liquid chromatography, ion exchange chromatography, gel filtration chromatography, electrophoresis and ultrafiltration etc. are utilized worldwide for end product purification.[5] These methods are traditionally being followed since years and are common for all types of enzymes, but they are time consuming and cause high loss of enzyme yield. Since commercial applications don't demand extremely high degree purity of enzymes; this necessitates the utilization of rapid purification protocols to keep the process economical. Biochemical properties of enzyme are also studied to understand the nature and its functionality. Tannase is reckoned as one of the costlier enzymes owing to its stupendous bio-catalytic potential and utilization in multitude of applications in food, feed, pharma, chemical and brewing industries.[6] One of the well-recognised applications of tannase is in Gallic acid production.[7] Tannase specifically acts upon ester & depside linkages in hydrolysable tannins thus giving off glucose and gallic acid.[1] Gallic acid occurs innately in wide range of herbs, fruits and in various processed beverages.[8] Over the years, gallic acid has emerged as an immensely valuable molecule owing to its wide ranging therapeutic and industrial applications.[9–10] The yearly requirement of Gallic acid for diverse applications is 8000 tons across the world.[11] Chemically, its production is accomplished by acid hydrolysis of tannins and tannic acid, wherein enormous amount of toxic effluent is released leading to environmental problems. This approach is unfavourable owing poor yield and substandard purity profiles of gallic acid produced.[10,12] Alternatively, gallic acid synthesis may also be accomplished via microbial/enzymatic fermentation approach.[13–15] Hence, biological method provides a greener solution. The bacterial tannase epitomizes an impressive potential for industrial utilization.[16] A detailed perusal of literature reveals that the original optimization studies

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on bacterial tannase for gallic acid synthesis from fruit waste are scanty.

The innate capability of bacterial tannase in hydrolysing natural tannins may play an effective role in gallic acid synthesis from fruit waste tannins. Optimization of gallic acid production has also been reported in previous studies.[10] Optimization of important process parameters play a significant role in minimizing the production cost.[17] Thus, considering these facts current study involves optimization of gallic acid synthesis by exploiting the tannase of *Bacillus haynesii* SSRY4 MN031245 for gallic acid synthesis from fruit waste.

Materials & Methods

Purification and characterization study was done to understand the properties and nature of bacterial tannase obtained from *Bacillus haynesii* SSRY4 MN031245. Purification strategies were employed in a step-wise manner to carry out the easy recovery of enzyme in purified form.

Microorganism and Tannase Production

Tannase-producing bacterium *Bacillus haynesii* SSRY4 MN031245 was employed for extracellular tannase production.[18] Fermentation was carried out in 1000 ml minimal media (KH_2PO_4 (0.5 g), K_2HPO_4 (0.5 g), Glucose (5 g), $NaNO_3$ (6 g), KCl (5 g), $MgSO_4$ (0.5 g) and Tannic acid (25 g), pH 5.5. An inoculum size of 5% (v/v) was used for inoculation. Incubation of the media was done at 37°C for 72 h at 150 rpm.

Tannase Activity and Protein Quantification

Tannase activity (U/ml) was determined through the rhodanine assay method.[19] Protein estimation was accomplished using Bradford method.[20]

Enzyme Purification

Ammonium Sulphate Precipitation and Dialysis

The crude extracellular tannase was precipitated with ammonium sulphate in the primary step of purification to achieve 40–90% saturation.[21] The precipitated proteins obtained by centrifugation were dissolved in 0.05 M citrate buffer (pH-5.5). The precipitates were further dialysed against the same buffer using 14,000 Molecular weight cut-off membrane (Sigma-Aldrich, India).

DEAE-Sephadex Anion Exchange Chromatography

A manually packed DEAE-Sephadex A-50 column (1.5 × 10 cm) was equilibrated with 0.05 M citrate buffer (pH 5.5). The dialyzed enzyme sample was loaded into column. Elution was commenced initially with 0.05 M citrate buffer (pH-5.5). Thereafter elution was done sequentially using a linear gradient (0.3–1 M NaCl) of 0.05 M citrate buffer (pH 5.5). Elution was done at a flow rate of 1 ml/min. Eluted fractions were collected and monitored for protein content and enzyme activity.[19,20] The fractions showing highest enzyme activity were processed for further characterization.

Characterization of Purified Enzyme

The purified tannase obtained from *Bacillus haynesii* SSRY4 MN031245 was characterized in terms of molecular weight, kinetic constants, temperature & pH stability.

Molecular Weight of Tannase

SDS-PAGE analysis was carried out to determine the molecular weight of purified enzyme.[22] Visualization of protein bands was accomplished through staining with Coomassie blue.

Temperature Optima and Thermostability

The study concerning temperature optima was accomplished by incubation of the reaction mixture at different temperatures ranging (25 – 65°C).Thermal stability was determined by incubation of the purified enzyme in the temperature range (25 – 65°C) for 4 hours. Enzyme assay was performed to determine the relative tannase activity.

pH Optima and Stability

The study concerning pH optima was accomplished by incubation of the reaction mixture at different pH ranging from 3.0 to 8.0. pH stability was determined by incubation of the purified enzyme in different buffers of pH range (pH 3.0 to 8.0) for 4 hours. Relative tannase activity was determined.

Determination of Kinetic Constants

Purified enzyme were estimated for K_m & V_{max} by incubating the enzyme with different concentrations of methyl gallate ranging (0.5 mM – 5 mM) under standard assay conditions. All the experiments were done in triplicates.

Application Study of Tannase

The application study of tannase involving potential gallic acid synthesis from the hydrolysis of fruit waste tannins was carried out by employing partially purified tannase from *Bacillus haynesii*

SSRY4 MN031245. Confirmation of gallic acid production from the selected substrate using partially purified enzyme was carried out against reference sample through analytical techniques such as FTIR and HPLC.

Tannin Extraction from Tannin Containing Fruit Waste

Tannin containing fruit-wastes including black plum seeds, tamarind seeds, mango seeds, pomegranate peel and tea waste from kitchen were collected and dried in oven. The dried fruit wastes were ground to fine powder. Tannin extraction was carried out from powdered substrate with tap water through Soxhlet extraction. After extraction, suspended solids were removed through filtration by muslin cloth.

Quantitative Estimation of Total Tannins

Tannin content of each extract was determined using protein precipitation method.[23] The tannin content was expressed in terms of mg/gm of the sample. The substrate showing highest tannin content (mg/g) was selected for gallic acid synthesis.

Optimization of Parameters for Gallic Acid Synthesis

Gallic acid synthesis was accomplished by enzymatic hydrolysis of hydrolysable tannins. Partially purified tannase from *Bacillus haynesii* SSRY4 MN031245 was employed for the study. Partially purified tannase (16.80 U/mL) was reacted with crude tannin extract for the hydrolysis reaction. The optimization of process parameters such as volume of tannin extract (5–15 mL), enzyme aliquots (1–10 mL) and incubation period (12–72 hours) were accomplished by One Factor at a Time approach to facilitate the maximum gallic acid synthesis. Incubation of the samples was done at 40°C and agitation speed of 140 rpm. Samples were withdrawn after every 12 hours for confirmation and quantitative determination of gallic production.

Detection and Quantification of Gallic Acid Synthesis

Confirmation of gallic acid production and its quantitative determination was done through FTIR Spectroscopy and RP-HPLC. The confirmation of the gallic acid production was verified against the standard gallic acid.

FTIR Spectroscopy

Potassium bromide (KBr) disc technique was employed for qualitative analysis of the enzymatic reaction. Since the KBr has no absorption in the fundamental region of IR spectrum, only the spectrum of sample is obtained. The spectral range used in FTIR analysis was 4000–450 cm^{-1}.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC) Analysis

Quantitative estimation of gallic acid was accomplished with HPLC system (Shimadzu, Japan) using optimized chromatographic conditions:

- **Stationary Phase:** C_{18}, 250 × 4.6 mm, 10 µ particle size, Phenomenex
- **Elution mode:** Isocratic elution mode (90:10 v/v)
- **Mobile phase:** Solvent A was water and Solvent B was acetonitrile (B), both containing 1% acetic acid
- **Detector:** UV
- **Absorption maxima:** 275 nm
- **Column Temperature:** 35°C
- **Flow rate:** 1 ml/min
- **Injection volume:** 25 µl
- **Diluent:** Citrate buffer

The suspension collected from hydrolysis reaction was analysed for gallic acid synthesis in accordance with the chromatographic conditions. The presence of Gallic acid in the sample was confirmed by superimposition of peaks of standard Gallic acid (Sigma Aldrich) and sample.

Results and Discussion

The extracellular tannase obtained from *Bacillus haynesii* SSRY4 MN031245 in the fermented broth was purified step-wise using different strategies. Purified tannase was characterized to identify the biochemical properties and understand the kinetics of the enzyme.

Purification and Characterization of Tannase

Different purification strategies were employed in a step-wise manner to carry out the easy recovery of enzyme in purified form. Fractional precipitation with ammonium sulphate was done from 40% to 90% saturation. The non-enzyme proteins were eliminated and tannase was precipitated at 70% saturation with 16.80 ± 1.06 U/mL of enzyme activity. About 60% of the total tannase was recovered by 70% saturation with ammonium sulphate. Tannase from *B. cereus*, *E. faecalis*, *Rhodococcus* sp., *Bacillus subtilis* KMS2-2 and many others have been partially purified at different saturated concentrations using ammonium salt.[24,25] The partially purified enzyme was further

subjected to ion exchange chromatography using DEAE-Sephadex A-50 column. The proteins were eluted with a NaCl gradient (0.3–1.0 M). The elution profile of tannase from DEAE Sephadex A-50 column showed one major peak (Fig. 1). Fraction 6 gave the highest tannase activity of 34.5±1.02 U/mL. The eluted fractions gave a purification fold of 42.0 with 36.30% of enzyme yield (Table 1). Purified tannase obtained from *Bacillus haynesii* SSRY4 MN031245 was further characterized and biochemical properties of the enzyme were studied. SDA-PAGE analysis of the purified elute obtained from Sephadex A-50 column showed single band of 35 kDa mol. wt. indicating the homogeneity and monomeric nature of the protein (Fig. 2). A single band of 31 kDa, 40 kDa, 46.5 kDa, 43 kDa and 45 kDa was also observed for

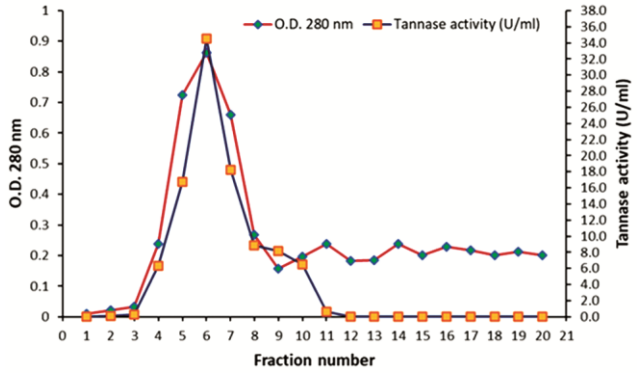

Fig. 1 — Elution profile of tannase from *Bacillus haynesii* SSRY4 MN031245 in DEAE-Sephadex anion exchange chromatography

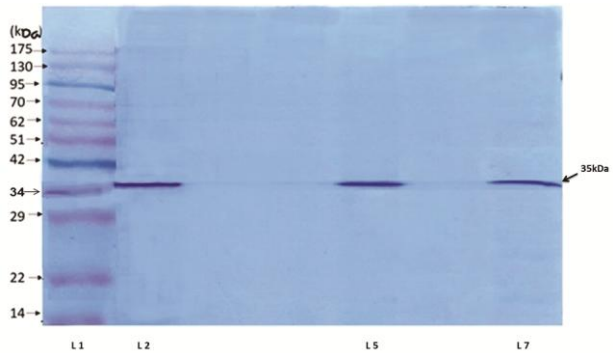

Fig. 2 — SDS-PAGE of purified tannase of *Bacillus haynesii* SSRY4 MN031245 (L1- Protein Ladder), (L2, L5, L7- Purified protein sample Lanes)

bacterial tannases in previous reports.[26–29] The study of temperature optima revealed that purified tannase exhibited maximum enzyme activity at 40°C which was similar to tannase from *Bacillus Cereus.*[24] The optimal temperature for most bacterial tannase has been documented in between 30°C and 40°C which is also in harmony with the present investigation. Increase in the temperature above 40°C resulted in decline in the enzyme activity. The decrease in enzyme activity upon increasing the temperature is attributed to increased rate of denaturation of enzyme. The enzyme activity also observed a decline below 40°C possibly due to insufficient energy to catalyse the enzymatic reaction. Another important finding of this study is that the enzyme retained 90% of its activity at 40°C and 23% activity at 60°C which makes it promising for industrial applications. The thermal stability of purified enzyme observed in present study is consistent with the previous reports.[30] The study of pH optima revealed that purified enzyme exhibited maximum activity at pH-5.5. Below or above pH 5.5 enzyme activity observed a decline probably due to deformation in the enzyme structure. The optimum pH for most bacterial tannases had also been recorded in the acidic range which is also in agreement with our study. Another important observation of this study is that purified enzyme was able to retain more than 80% of its activity at pH 5.5. K_m and V_{max} values determined through Line weaver–Burk reciprocal plot (Fig. 3) were recorded as 1.86 mM and 188.67 µmoles/ml/min, respectively. Similar kind of substrate affinity (K_m) values 1.6 mM was recorded for bacterial tannase in previous study with methyl gallate as substrate.[31]

Application Study of Tannase

The interpretation of findings revealed that different substrates examined showed wide variations in terms of their tannin content. It was observed that a substrate: water ratio of 1:3 proved to be most suitable for tannin extraction from all the substrates evaluated. Black plum seeds were reported to exhibit highest tannin content (196.18 mg/g) amongst all the substrates tested (Table 2). The tannin content of

Table 1 — Purification profile of tannase from *Bacillus haynesii* SSRY4 MN031245

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude Enzyme	11190	610	18.24	1	100
Ammonium sulphate Precipitation	6714	30.84	217.70	11.93	60
DEAE-Sephadex anion exchange chromatography	4062	5.30	766.41	42	36.30

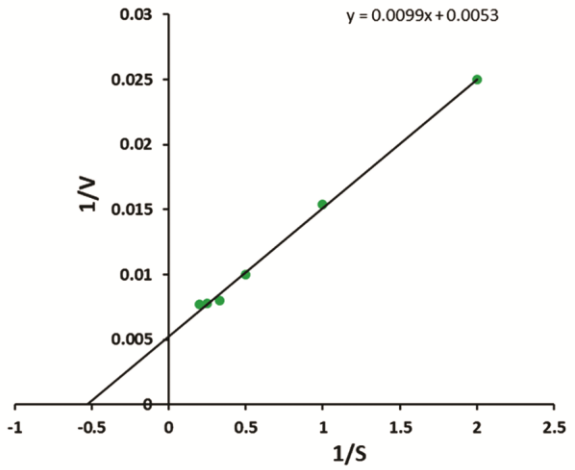

Fig. 3 — Lineweaver-Burke plot for purified tannase from *Bacillus haynesii* SSRY4 MN031245

Table 2 — Tannin content of various substrates used (Substrate: water ratio-1:3)

Substrate	Scientific Name	Part used	Tannin Content (mg/g)
Black plum	*Syzygium cumini*	Seed	196.18 ± 0.02
Tamarind	*Tamarindus indica*	Seed	133.25±0.03
Pomegranate	*Punica granatum*	Outer peel	82.62 ± 0.05
Mango	*Mangifera indica*	Seed	0.23 ± 0.03
Tea	*Camellia sinensis*	Tea waste	73.07 ± 0.01

black plum seeds recorded in our study is comparable to that reported in the previous reports.[32] Tannin estimation results revealed that crude tannin extract from black plum seeds exhibited highest tannin content of 196.18 ± 0.02 mg/g amongst all the substrates tested. Hence, it was selected as substrate for gallic acid synthesis.

Analysis of Hydrolysis Reaction

Confirmation of Gallic acid production from the enzymatic reaction was carried out against standard reference sample. Reaction samples were taken for analysis and were appropriately diluted. All the experiments were performed in triplicates. Gallic acid synthesis was confirmed through (FTIR Spectroscopy and RP-HPLC.

FTIR Analysis

The FTIR spectra of gallic acid was measured from spectral range of 4000–450 cm^{-1} The principal IR absorption peaks of gallic acid at 1698.79 cm^{-1} corresponds to stretching vibration of OH group and carbonyl group), which indicated the occurrence of carboxyl group in the gallic acid. Three peaks

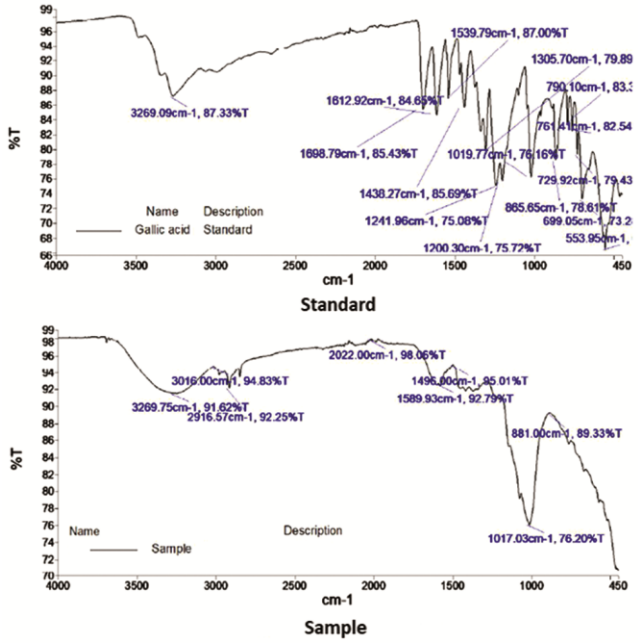

Fig. 4 — FTIR analysis

observed at 1612.92 cm^{-1}, 1539.79 cm^{-1}, and 1438.57 cm^{-1} are typical stretching vibrations of C–C bonds in an aromatic ring. Various peaks in 1300–1000 cm^{-1} region could be assigned to the stretching vibration of C–O bond and bending vibration of O–H bond of gallic acid such as 1241.96 cm^{-1}, 1200.30 cm^{-1}, and 1019.77 cm^{-1}. The sample correlates with the bonding patterns of commercially available gallic acid (Fig. 4). All the spectrum peaks revealed that corresponding peaks of standard gallic acid are present in the sample spectra also. The results in the present study are supported by the results obtained in previous study.[33] Hence production of gallic acid could be predicted by this analysis.

HPLC Analysis and Optimization of Gallic Acid Synthesis

Bioconversion of tannins to gallic acid was done through hydrolysis reaction between rude tannin extract of selected substrate i.e., Black plum seeds and partially purified tannase from *Bacillus haynesii* SSRY4 MN031245. The suspension collected from hydrolysis reaction was analysed by RP-HPLC equipment for estimation of Gallic acid in accordance with the chromatographic conditions. It was observed that 10 ml of crude tannin extract of the selected substrate mixed in 0.05 M citrate buffer when treated with 5 ml of partially purified enzyme for 24 h at 40°C on a rotary shaker at 140 rpm resulted in maximum synthesis of gallic acid. The superimposition of peaks confirmed the

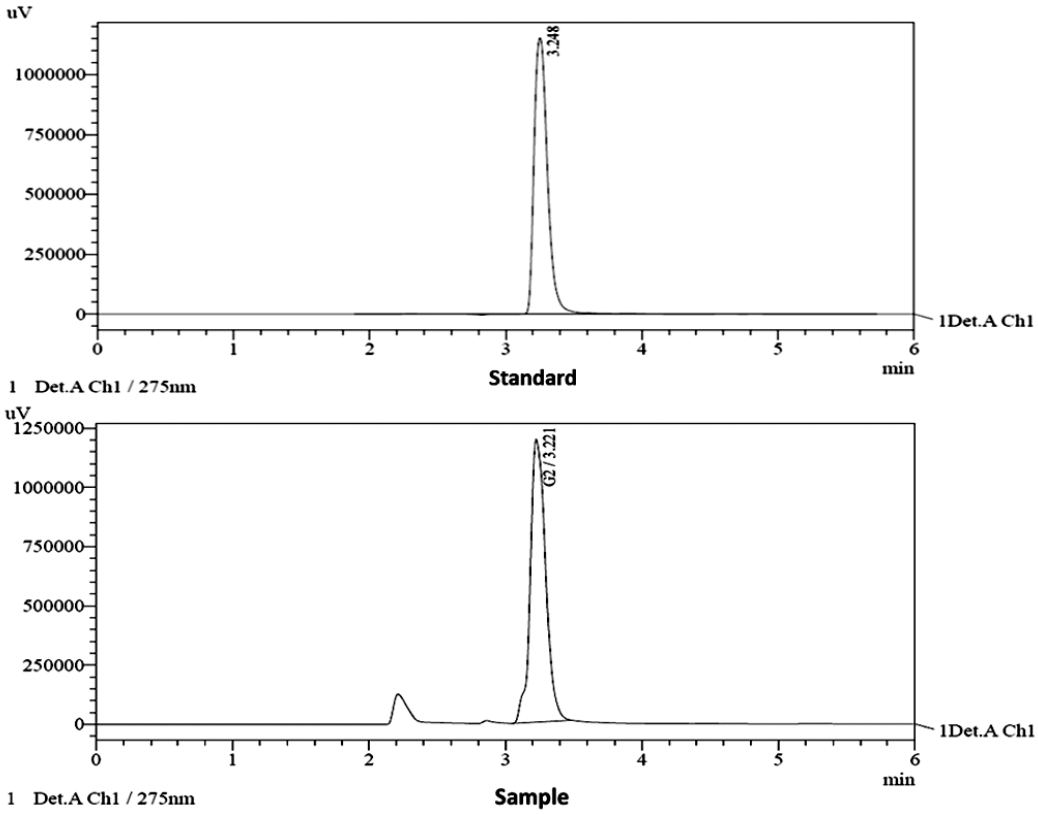

Fig. 5 — HPLC analysis

presence of Gallic acid in the fermented sample at the retention time of 3.22 minutes compared to retention time of 3.24 minutes of standard Gallic acid (Fig. 5). Tannin hydrolysis and the accumulation of gallic acid can be identified clearly. In the present work, the HPLC chromatogram of the sample harvested after 24 hours exhibited a peak area value of 9317072. The peak area value was used in regression equation to calculate the yield of Gallic acid produced. Following the optimized chromatographic conditions and using the regression equation; quantitative HPLC analysis revealed a gallic acid yield of 20.304 mg/ml in the suspension collected from hydrolysis reaction. Arshad *et al*. also reported 16.66 mg/g gallic acid from black plum seeds using *Aspergillus oryzae* in solid-state fermentation under optimized physicochemical conditions, 1:3 substrate: moisture ratio, 30°C, 96 h incubation period and pH 5.5.[32] The Gallic acid production in present study is relatively higher than gallic acid production reported by Goel *et al*. and Aguilar-Zarate *et al*. who recorded 0.28 mg/mL, 8.63 g/L yield of gallic acid respectively through HPLC analysis.[34,35]

Conclusions

Purification of tannase from *Bacillus haynesii* SSRY4 MN031245 was successfully carried out by multi-step strategies which yielded 36.30% enzyme with a purification fold of 42.0. An important feature of the enzyme was its low K_m value of 1.86 mM which makes it immensely suitable for applications in the food-processing industry. Another important finding of this study was its low molecular weight of 35 kDa, thus signifying its utilization in various industrial bioprocessing applications demanding the low molecular weight enzymes. Partially purified tannase of *Bacillus haynesii* SSRY4 MN031245 was able to synthesize significant amount of gallic acid (20.304 mg/ml) from fruit waste. The present investigation gave a fair amount of idea about bacterial tannase and its biochemical properties, which may explore new horizons of research on tannase. However, extensive research studies would also be required to enhance the tannin hydrolysis rate. Large scale studies would also be required to assure improved process control for increased activity of tannase. Natural/waste resources that are readily available should be tested for tannase and Gallic acid

production at large scale. This may minimize the overall production cost and can have a positive impact on environment.

Conflicts of interest

None

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