Abstract of thesis titled

Tannase from *Penicillium charlesii*: Process Optimization, Purification, Characterization, Scale up and Industrial Applications

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SHAILENDRA RAGHUWANSHI

DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF DELHI SOUTH CAMPUS
BENITO JUAREZ ROAD
NEW DELHI-110021
INDIA
ABSTRACT

Tannase (Tannin acyl hydrolase E.C. 3.1.1.20) is a ubiquitous enzyme of considerable physiological and industrial significance and is found in plants, animals and microorganisms. This hydrolytic enzyme catalyzes the hydrolysis of ester and depside bonds of hydrolysable tannins, releasing glucose and gallic acid.

Tannase exhibits the potential for both hydrolytic and synthetic reactions under aqueous and organic solvent systems. Due to the versatile catalytic nature of tannase, the enzyme is used for various applications in different industries like food, feed, beverage, brewing, pharmaceutical, chemical, detergent, cosmetic, textiles, leather and many others. Therefore, realizing the importance of tannase, the present investigation was planned and executed with the following main objectives.

OBJECTIVES

Section I : Screening and selection of potential tannase producer/s
Section II : Identification of selected micro-organism/s
Section III : Process optimization for tannase production
Section IV : Scale up and its optimization in fermentor
Section V : Purification
Section VI : Immobilization
Section VII : Characterization and kinetic studies
Section VIII : Potential industrial applications

To achieve these objectives, detailed investigation was undertaken by following appropriate methodology and the main results obtained are presented below.

RESULTS

One hundred fifty fungal strains and one hundred fifty bacterial isolates were screened for tannase production on tannic acid agar plate this finally resulted in the selection of one hundred and three tannase positive fungal cultures and
only five positive bacteria isolates which showed hydrolytic zones on tannic acid agar plates. Subsequently, quantitative analysis of tannase production resulted in the selection of, six fungi i.e. Aspergillus terreus, A. flavus, Penicillium sp. SS-1, Penicillium sp. (SR-75), P. chrysogenum and P. crustosum. Final selection was based on pH–temperature tolerance and stability and organic solvent stability of the tannase produced. This resulted in the selection of Penicillium sp. SS-I as the most potent tannase producer which was identified as Penicillium charlesii, on the basis of 18s rRNA and 600 bp analysis.

This Penicillium sp. produced 4.96 IU/ml tannase when 1.0% of tannic acid was present in the production medium (1.0% tannic acid; 0.6% NaNO₃; 1.52 g/l KH₂PO₄; 0.05 % KCl; 0.52% MgSO₄.7H₂O; FeSO₄.7H₂O, ZnSO₄.7H₂O and Cu(NO₃)₂.3H₂O (in traces); pH 5.0 and inoculated with 5x10⁶ spores/50 ml). However in absence of tannic acid tannase production was not detected, it confirmed that Tannase production was inducible in nature. Further optimization by one variable at a time strategy resulted in 35.65 IU/ml of tannase titres in medium containing tannic acid (2%); Sucrose, 0.8 %; NaNO₃, 0.8 %; Tween-80, 0.3%; MgSO₄.2H₂O, 0.08%; pH 5.0, inoculated with 5x10⁷ spores/50 ml and incubated at 30°C, 150 rpm. This yield of tannase is nearly 7.18 fold higher as against the initial titres, i.e. 4.96 IU/ml under initial unoptimized conditions.

Use of response surface methodology (RSM) to further enhance tannase production wherein Tannic acid, Sucrose, NaNO₃ and MgSO₄ were the significant factors, resulted in the production of 58.14 IU/ml of tannase in 60 h in medium containing Tannic acid, 2%; Sucrose, 1.0 %; NaNO₃, 1.0 %; Tween-80, 0.3%; MgSO₄, 0.08%; pH 5.0, inoculated with 5x10⁷ spores/50 ml and incubated at 30°C, 150 rpm. This is 11.72 fold higher as against the initial tannase titres under unoptimized conditions (4.96 IU/ml) and 1.63 fold higher as against the titres obtained after optimization by one variable at a time approach (35.65 IU/ml). The production was further scalable to 1L production medium volume contained in 5L conical flask where 58.09 IU/ml of tannase titres were obtained. The other strategy followed for tannase production was solid state fermentation (SSF).

Production in SSF initially resulted in 35.49 IU/gds of tannase using 10.0 g of wheat bran supplemented with 10% tannic acid, 20 ml distilled water
(pH adjusted to 5.0) to obtain a moisture ratio of 1:2, inoculated with $1 \times 10^6$ spores/gds (gram dry substrate) and incubated at 30°C for 72 h.

**Optimization of tannase production in SSF by one variable at a time** resulted in 182.63 IU/gds of tannase when Jamun dried leaves (Syzygium cumini, 10.0 g) supplemented with 5% tannic acid, moistened with salt solution [0.8% NaNO$_3$, 1.52 g/l KH$_2$PO$_4$, 0.52 g/l KCl, 0.80 g/l MgSO$_4$.7H$_2$O, FeSO$_4$.7H$_2$O (trace), ZnSO$_4$.7H$_2$O (trace), Cu(NO$_3$)$_2$.3H$_2$O (trace) and 1.0% sucrose] at a moisture ratio of 1:3 and water activity ($a_w$) 0.92 was inoculated with $1 \times 10^7$ spores/gds and incubated at 30°C for 72 h. This is 5.16 fold higher as against the initial unoptimized yield. Further optimization by RSM using Tannic acid, moisture ratio, sodium nitrate and inoculum density (significant factors) resulted in 324.37 IU/gds of tannase in medium containing: Jamun dried leaves (Syzygium cumini), 10.0 g; tannic acid concentration of 5.0%; moisture ratio of 1:2.5; NaNO$_3$ concentration of 1.0%; inoculum density of $1 \times 10^7$ spores/gds; water activity ($a_w$) 0.92 and incubated at 30°C. This final tannase yield is 9.16 fold higher as compared to initial unoptimized yield. The SSF system was scalable to 1 kg solid substrate size.

Further scale up of tannase production was now attempted in fermentors. Scale up and optimization was first attempted in a 30 L fermentor resulting in 64.32 IU/ml in 54 h at 20% DO saturation, 200 rpm and aeration of 0.5 vvm. Further scale up and optimization in a 300 L fermentor resulted in 64.19 IU/ml in 54 h when fermentation was carried out at optimized conditions for 30 L bioreactor. For further increase in tannase titres, fed batch was attempted with Tannic acid (1.0%) after 36 h of incubation. This finally resulted in 68.45 IU/ml of tannase in 54 h of incubation at 30°C, 200 rpm, 20% DO saturation and aeration of 0.5 vvm.

**For purification, concentration** of the tannase rich crude culture broth via ultrafiltration using 50 kDa membrane cartridge resulted in 5.21 fold purification with 96.25% yield. Precipitation with ammonium sulphate (0-80%) resulted in 7.14 purification fold and 91.20% yield. Absolute purification carried out using gel filtration (Sephadex G-100) chromatography which resulted in 40.6 fold purification and 63.4% recovery. The purified tannase had a molecular weight of 70±1 kDa on SDS-PAGE with a pl of 4.3.
Different strategies for immobilization of this tannase showed an immobilization efficiency of 76.45% on DEAE cellulose by conventional immobilization procedure. Immobilization efficiency of 70.46% was obtained by covalent binding using chitosan followed by cross linking of tannase by glutaraldehyde.

A novel method of carrier free immobilization was achieved by synthesis of cross linked enzyme aggregates (CLEAs) using ammonium sulphate as precipitant with glutaraldehyde as crosslinking agent and Sodium taurocholate as surfactant which resulted in 83.39% relative tannase activity. Reusability of the three forms with respect to tannic acid hydrolysis showed that the immobilization on DEAE Cellulose was stable 100% upto the 3rd cycle, covalent binding on chitosan was stable upto the 5th cycle. However, the immobilization through CLEA are 100% stable even upto the 8th cycle. Results clearly indicate that covalent and CLEA forms of immobilized tannase are much more efficient as against conventionally immobilized tannase on DEAE Cellulose.

All the three tannase forms (crude, purified and immobilized) had optimal activity at acidic pH 5.0 with activity between pH 3.0-9.0. Optimal tannase activity was observed at 40°C for all these forms with activity between 20-80°C. The immobilized tannase was more thermostable with nearly 30% RA after 1h of incubation at 80°C. This $P$. charlesii tannase was stable in a wide range of organic solvents with maximum stability in ethyl acetate for crude (81.69%) and immobilized (86.4%) tannase even after 48 h of incubation. Slight increase in tannase activity was obtained in presence of Sodium taurocholate with 102.0 %, 109.0 % and 110.0 % RA for crude, purified and immobilized forms respectively. Tannase activity was completely inhibited by N-Bromosuccinimamide and PMSF. Metal ions and chelating agents did not stimulate tannase activity. This tannase preferentially hydrolyzed tannic acid (95%) tested followed by propyl gallate (76%), and methyl gallate (73%). Results of the kinetic studies showed that by plotting Lineweaver Burke plot with tannic acid as substrate has a $K_m$ of 0.18 mM, $V_{max}$ of 166.7 μmole/ml/min and $K_{cat}$ of 15.43 sec$^{-1}$. This tannase also exhibited stability for one year in all three crude, purified and lyophilized forms at 4 and 30°C.
This tannase of *P. charlesii* was evaluated for various industrial applications. Tannase carry out 92.30% conversion of tannic acid to gallic acid. Gallic acid production was successfully scaled up to 2.5 L reaction volume. Successfully methyl gallate (82%); propyl gallate (93); butyl gallate (51%); amyl gallate (32%) and ethyl gallate (63%) were synthesized using this tannase.

Black tea infusion treated with tannase showed that both epigallocatechin gallate and epicatechin gallate of tea catechins were hydrolysed by this enzyme into epigallocatechin and epicatechin, respectively, accompanied by 11.2 fold (CTC tea) and 10.29 fold (Kangra orthodox tea) increase in gallic acid concentration. The tannase treated tea infusion showed reduction in tea cream formation, and an increase in antioxidant activity to 1.73 and 1.61 fold respectively. However, there was no change in the content and concentration of volatile compounds. Moreover, there was an improvement in the quality of tannase treated black tea infusion in relation to color, brightness, strength and flavor as against control.

This tannase efficiently degraded tannin in wheat straw which improved the digestibility and nutrient availability in ruminants. Tannin free fermented biomass led to a significant decrease in cell wall constituents viz, acid detergent fiber, neutral detergent fiber, hemicellulose, lignin and cellulose up to 20.26, 19.47, 17.86, 37.81 and 20.45% respectively after 10 days of incubation. The tannin free myco-straw showed a significant 1.27 fold increase in crude protein content along with lower L/C and lower C/N ratio as compared to control wheat straw. Thus, showing potential for the bioconversion of lignin rich wheat straw into high energy cattle feed.

This tannase was also evaluated for its potential for tannery effluent treatment, results of the present study revealed that; there is a considerable reduction in the values of important parameters. The tannery effluent had color equivalent to 4439 U. The treatment of the effluent with the enzyme reduces the color to 3241 U. The pH value of the raw effluent increased from 4.3 to 6.0. Significant reduction in the values of TDS, TSS, COD, chloride and tannin by the enzyme was observed. Hence, this tannase can be successfully exploited for the treatment of tannery effluent.
This tannase also effectively remove tea and betel stains proving it to be a good detergent additive.

It is evident from the results obtained that the tannase from *Penicillium charlesii* has excellent catalytic properties which make it suitable to carry out several commercially important industrial applications. Also, the tannase production from this strain is amenable to process optimization and can be scaled up to large volumes thereby indicating its commercial value and importance.

SHAILENDRA RAGHUWANSHI
Candidate

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**Prof. R. K. Saxena**
Department of Microbiology
University of Delhi South Campus
Benito Juarez Road
New Delhi-110021

**Prof. J. S. Virdi (Head)**
Department of Microbiology
University of Delhi South Campus
Benito Juarez Road
New Delhi-110021