Full Length Research Article

OPTIMIZATION OF CULTURE CONDITIONS FOR TANNASE PRODUCTION BY *STREPTOMYCES SP.*

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ABSTRACT

Tannin Acyl Hydrolase (E.C 3.1.1.20) commonly referred as tannase is an industrially important enzyme which is mainly used in the food, chemical, pharmaceuticals and beverage industry; it is produced by number of microbes, animals, and plants. In this study production of tannase was investigated using agro-residue, *Azadirachta indica* leaves as a substrate of hydrolysable tannin by potent tannase producer isolate *Streptomyces sp.*SKA1. Optimization of culture conditions for maximum tannase production included studying the effect of age of inoculum, inoculum size, incubation period, incubation temperature, pH, concentration of agro-residues, concentrations of carbon and nitrogen sources on enzyme activity. The optimum culture conditions determined were Inoculum age-5 days (120 hrs), inoculum volume -6 %, incubation period-5 days (120 hrs), incubation temperature - 30°C, pH - 7, agro-residue concentration – 4%, as a substrate, starch-1% (w/v) as a carbon source and casein- 0.03% (w/v) as a nitrogen source for maximum tannase activity.

INTRODUCTION

Tannins are water soluble polyphenols naturally occurred as secondary metabolites in higher plants (Amitsinh *et al.,* 2014). It has been considered as the fourth abundant constituents after cellulose, hemicelluloses, and lignin (Rana and Bhat, 2005). Tannins are considered as nutritionally undesirable because they form complexes with protein, starch and digestive enzymes and cause reduction in nutritional value of food (Amitsinh *et al.,* 2014). Tannins are present in large number of feed and forages. The formation of complexes of tannins with nutrients, such as carbohydrates, proteins and minerals has negative effects on their utilization. High concentration of tannins depresses voluntary feed intake and digestive efficiency. The nutrient value of tanniferous feed may be enhanced by various detanification procedures viz., physical, chemical and biological. In biological treatment; various tannase producing microbial strains have been tried for reduction of tannin content and nutritive enhancement of treated material (Enemuor *et al.,* 2009). Moreover, tannin found in industrial effluent water may also cause environmental problems. Therefore, it is very necessary to optimize the production of tannase from microbial sources.

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Tannic acid hydrolysis can be carried out by acid or alkali or microbial tannase. The enzymatic hydrolysis has advantage over the other methods because it is less energy intensive and less polluting (Hadi *et al.,* 1994). Tannase (Tannin Acyl Hydrolase, E.C 3.1.1.20) catalyses the hydrolysis of ester and depside bonds in hydrolysable tannins, as tannic acid, releasing glucose and gallic acid. Tannase was discovered accidently during the extraction of gallic acid from soluble tannins (Lekha *et al.,* 1993).Tannase cleaves the ester linkages between galloyl groups present in various compounds, such as epigallocatechin and epigallocatechin gallate that are present in green tea leaves (Lekha and lonsane 1997).Tannase find the applications in food, feed and beverage industries, it is used as a clarifying agent in wines, juices and beer. Most of the commercial applications of tannase are in the manufacturing of instant tea. The enzyme has applications in the treatment of tannery effluents and pretreatment of tannin containing animal feed. One of the major applications of tannase is the production of gallic acid, which is used for the manufacture of an antimalerial drug "Trimethoprim". Gallic acid is also used as a substrate for chemical and enzymatic synthesis of propyl gallate, which is used as an antioxidant in fats and oils (Hernandez *et al.,* 2006). Tannase has been isolated from number of microorganisms like fungi, bacteria and yeast, but there is a constant search for tannase with more desirable properties for its commercial applications. It is reported that the introduction of actinomycetes starter accelerates the

fermentation which significantly effects on the bioactive compounds such as tannins (Nurleni Kurniawati *et al.,* 2016), the tannin polyphenol in soil influence microbial growth. In non tannin soil, bacterial counts outnumbered fungal and actinomycetal count. The sequence of dominance in tannin soil followed the order: fungi<actinomycetes<bacteria. (Natrajan Hemlatha *et al.,* 2012), Techniques for production of tannase have been extensively studied and commercial production of tannase is achieved using synthetic tannic acid. The industrial process makes use of chemical tannic acid for tannase production but this process involving synthetic substrates has adverse environmental consequences. Conventionally gallic acid is also produced chemically by acid hydrolysis of synthetic tannic acid and suffer from disadvantages like high cost to yield ratio and low purity (Swaran Nandini *et al.,* 2014). Therefore it is very necessary to design a process for the production of tannase which is more economical and environmental friendly. This has led to generating interest in searching natural source for tannic acid that can be effectively utilized by microbes. This effort may reduce the dependency on synthetic tannic acid. With this view studies on isolation, screening and production of tannase from *Streptomyces sp.*SKA1 was carried out using *Azadirachta indica* leaves as a potent source of hydrolysable tannin. Attempts were made to optimize the cultural conditions for maximum tannase production by the conventional one factor at a time methodology.

MATERIALS AND METHODS

Chemicals

All the chemicals and reagents were of analytical grade purchased from HI-MEDIA Pvt. Ltd., Mumbai, India

Microorganism

Fifty nine actinomycetal isolates were isolated from different sites like garbage, tea waste dump sites, tannery industry. Actinomycetal isolates that succeeded to grow in the presence of tannic acid supplemented starch casein agar media were tested for tannase production individually. The most active tannase producing actinomycetal isolate was identified as *Streptomyces sp.*SKA1 and was maintained on starch casein agar slants containing tannic acid and stored at 4°C and sub-cultured at monthly intervals. Qualitative screening for tannase producing *Streptomyces sp.* SKA1 isolate was carried out on starch casein agar plate supplemented with 1% tannic acid and 3% agar and recording the clear zone formed due to hydrolysis of tannic acid around the actinomycetal colony, as shown in Fig 1.

Preparation of spore suspension

Actinomycetal spore suspension was prepared by adding 10 ml of sterile saline to a fully sporulated and induced culture. The spores were dislodged using a sterile inoculation loop under strict aseptic conditions. The volume of 1 ml of the prepared spore suspension containing 2×10^8 spores was used as inoculum.

Mode of fermentation

Cultivation of actinomycetes was carried out by surface fermentation in 250 ml Erlenmeyer flasks using the starch casein medium supplemented with powder of *Azadirachta indica* leaves as a source of tannic acid autoclaved at 110ºC; for 20 minutes and then inoculated with prepared inoculum (1% v/v) and incubated for 120 hrs at room temperature (30°C). Samples were withdrawn at regular intervals of 24 hours and observed for tannase activity.

Tannase Assay

A calorimetric assay was used to determine tannase activity, based on measuring the residual tannic acid content after the enzymatic reaction (Mondal *et al.,* 2001).The reaction mixture consisted of 0.5 ml of the substrate tannic acid (1% (w/v) in 0.2 M acetate buffer at pH 5.5) and 0.5 ml of the enzyme extract, incubating at 30ºC for 30 min. The enzymatic reaction further continued by the addition of 2 ml of a bovine serum albumin solution- BSA (1 mg/ml), leading to the precipitation of the remaining tannic acid . The tubes were then centrifuged at 10,000 rpm for 10 min. at 4°C; the precipitate was dissolved in 2 ml of SDS-triethanolamine solution and absorbance was measure at 530 nm after addition of 1 ml of 0.13 M $FeCl_3$ reagent. One unit of tannase activity was defined as the amount of tannic acid hydrolyzed by 1 ml of enzyme per minute of reaction:

$$Abs_{530} = Abs_{control} - Abs_{test}$$

Optimization of factors affecting tannase activity

Optimization of inoculum age: To optimize the effect of different inoculum age on tannase production, starch casein broth was used for each particular inoculum age. Each flask contained 50 ml of the medium, inoculated with 2% of spore suspension (2×10^8 cfu/ml) of *Streptomyces sp.*SKA1 using different inoculum ages of 2, 3, 4----15 days. At the end of incubation period (5 days) tannase activity was assessed.

Optimization of inoculum volume: *Streptomyces sp.*SKA1 was grown on starch casein agar slants for 5 days at 30ºC. Spores were harvested and re-suspended in saline. Spore suspension was inoculated (2×10^8 cfu/ml) in 250 ml conical flask containing 50 ml of starch casein broth supplemented with powder of *Azadirachta indica* leaves as a source of tannic acid at 1% to 10% (v/v) and incubated for 5 days at 30°C. After incubation period enzyme activity for each flask was determined.

Optimization of different incubation periods: To evaluate the effect of different incubation periods on tannase production, the spore suspension (2×10^8 cfu/ml) of optimum inoculum size (5% v/v) was inoculated in each 250 ml flasks containing 50 ml of starch casein broth supplemented with powder of *Azadirachta indica* leaves as a source of tannic acid. The flasks were incubated at 30°C for different time periods ranging from 1 day to 10 days and checked for tannase activity.

Optimization of different incubation temperatures: This experiment was constructed to determine the optimum growth temperature at which maximum biosynthesis of the active agent occurred. The flasks containing starch casein broth supplemented with powder of *Azadirachta indica* leaves as a source of tannic acid were inoculated using the spore suspension (2×10^8 cfu/ml) of optimum inoculum size (5% v/v) and incubated at temperatures 20°C, 30°C, 37°C, 40°C, 42°C,

50°C, 60ºC. The tannase activity was assessed at each temperature.

Optimization of different pH: The effect of initial pH on tannase production was studied by adjusting the production medium at various levels of pH by 1 N HCL and 1 N NaOH solutions ranging from 4.0 to 10.0 before sterilization. After inoculation of spore suspension, the flasks were incubated at 30°C for 5 days and after incubation the tannase activity was assessed.

Optimization of different concentrations of agro-residues: Various concentrations of agro-residues, ranging from 1%-10% (w/v) concentrations were supplemented in the basal starch casein broth and the flasks were inoculated, incubated and assessed for tannase activity.
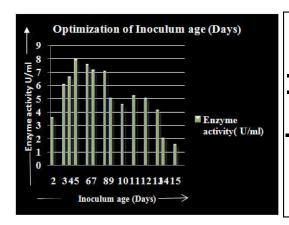
RESULTS AND DISCUSSION

Optimization of inoculum age: Maximum tannase activity was observed at inoculum age 5 days as it was detected by the highest tannase activity. In statistical analysis paired t-test was applied using Minitab 14.0 software. From the t-test, p-value was determined and was found 0.048 showing that the experiment was significant above 95% confidence level.

Optimization of inoculum volume: Data recorded in Figure 3, determined that maximum tannase activity by *Streptomyces sp.*SKA1was obtained with 6% (v/v) inoculum size concentration. The t-test and p-value were calculated. The experimental model was significant as the p-value was < 0.05 that is above 95% confidence level.

Figure 1. Actinomycetal isolate SKA1 showing zone of hydrolysis on starch casein agar plate supplemented with 1% tannic acid

Paired t-test and CI: Inoculum age (Days) and Enzyme activity (U/ml)
Paired t for Inoculum age (Days) - Enzyme activity (U/ml)

	N	Mean	St. Dev	SE Mean
Inoculum age	14	8.50000	4.18330	1.11803
Enzyme activity	14	5.21429	1.97171	0.52696
Difference	14	3.28571	5.63519	1.50607

95% CI for mean difference (0.03205, 6.53937)
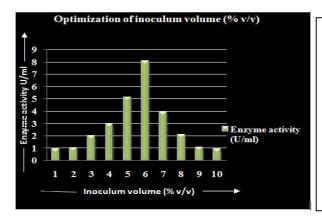T-Test of mean difference = 0 (vs. not = 0)
T-Value = 2.18 P-Value = 0.048

Figure 2. Optimization of inoculum age

Optimization of different carbon sources: Several carbon sources such as Lactose, Cellulose, Tannic acid, Mannitol, Raffinose, Glycerol, Starch, Arabinose, Maltose, Ribose, Xylose, Fructose, Sucrose and Glucose at concentration 1 % were supplemented to the culture medium. The flasks were inoculated, incubated and assessed for tannase activity.

Optimization of different Nitrogen sources: Several organic nitrogen sources such as Peptone, Yeast extract Beef extract; Urea, Casein, Glycine, Alanine, Aspargine as well as inorganic sources such as Ammonium sulfate, Ammonium chloride, Ammonium nitrate, Dipotassium hydrogen phosphate, Potassium nitrate at a concentration 0.03% were supplemented to the culture medium. The flasks were inoculated, incubated and assessed for tannase activity.

Optimization of incubation period: -After every 24 hours, tannase activity was observed till 10th day of incubation which was found to increase up to 5 [th] day, (8.50 U/ml) whereas it decreased from 6 [th] day (Figure 4).Decreased enzyme yield on prolonged incubation could also be due to reduced nutrient level of the medium. It has been reported that tannase activity decreases after reaching maximum level, due to inhibition or degradation of enzyme (Suseela R.G. *et.al* 1983) Deepanjali Lal *et.al,*2012 observed the maximum tannase activity (162U/ml) on 7 [th] day (168 hrs.) of incubation by using *Aspergillus niger* for tannase production. The t-test and p-value were calculated. The experimental model was significant as the p value was found to be 0.047, which was < 0.05 that is above 95% confidence level.

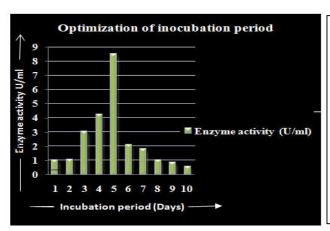**Paired t-Test and CI: Inoculum Volume (%v/v) and Enzyme activity (U/ml)**

Paired T for Inoculum Volume (% v/v) - Enzyme activity (U/ml)

	N	Mean	St. Dev	SE Mean
Inoculum Volume	10	5.50000	3.02765	0.95743
Enzyme activity	10	2.78900	2.31169	0.73102
Difference	10	2.71100	3.71790	1.17570

95% CI for mean difference: (0.05137, 5.37063)
T-Test of mean difference = 0 (vs. not = 0)
T-Value = 2.31 P-Value = 0.047

Figure 3. Optimization of inoculum volume

Paired t-Test and CI: Incubation period (days) and Enzyme activity (U/ml)

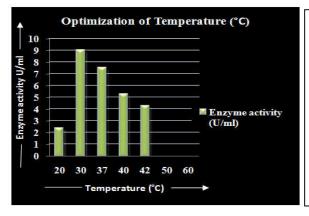Paired T for Incubation period (days) - Enzyme activity (U/ml)

	N	Mean	St. Dev	SE Mean
Incubation period	10	5.50000	3.02765	0.95743
Enzyme activity	10	2.38000	2.43572	0.77024
Difference	10	3.12000	4.28842	1.35612

95% CI for mean difference: (0.05225, 6.18775)
T-Test of mean difference = 0 (vs. not = 0)
T-Value = 2.30 P-Value = 0.047

Figure 4. Optimization of incubation period

Paired t-Test and CI: Temperature (ºC) and Enzyme activity (U/ml)
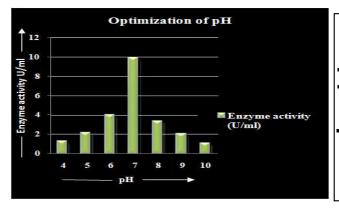
Paired T for Temperature (ºC) - Enzyme activity (U/ml)

	N	Mean	St. Dev	SE Mean
Temperature	7	39.8571	12.9927	4.9108
Enzyme activity	7	4.0586	3.5075	1.3257
Difference	7	35.7986	15.1703	5.7338

95% CI for mean difference: (21.7683, 49.8288)
T-Test of mean difference = 0 (vs. not = 0)
T-Value = 6.24 P-Value = 0.001

Figure 5. Optimization of temperature

Paired t-Test and CI: pH and Enzyme activity (U/ml)
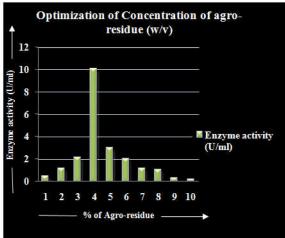
Paired T for pH - Enzyme activity (U/ml)

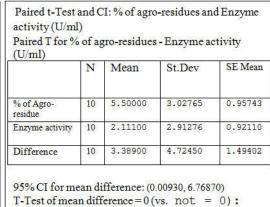|	N	Mean	St.Dev	SE Mean
pH	7	7.0000	2.16025	0.81650
Enzyme activity	7	3.38143	3.05843	1.15598
Difference	7	3.61857	3.81191	1.44077

95% CI for mean difference: (0.09314, 7.14400)
T-Test of mean difference = 0 (vs. not = 0)
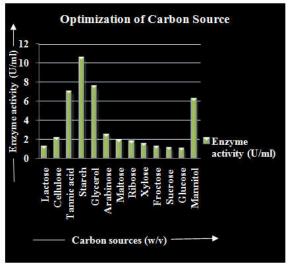T-Value = 2.51 P-Value = 0.046

Figure 6. Optimization of pH

Figure 7. Optimization of concentrations of agro-residues

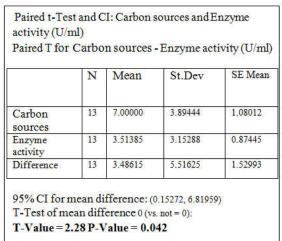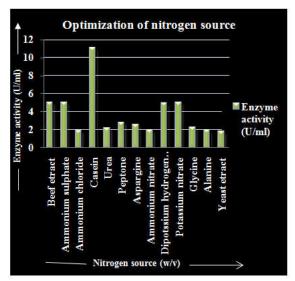**Figure 8. Optimization of carbon source**

Figure 9. Optimization of Nitrogen source

Optimization of temperature: Temperature plays an important role in microbial growth and enzyme activity. The obtained results showed maximum tannase activity (9.02 U/ml) at temperature of 30ºC. Above 30 ºC, tannase activity was decreased gradually (Figure 5).

This might be due to denaturation of some of the heat sensitive biochemical products produced during fermentation (Kar and Banerjee, 2000) whereas temperatures below 30 ºC, enzyme activity was lowered, this might be due to freezing of the protoplasmic membrane at lower temperature which causes inactivation of solute transport system in the cells (Papagianni,

2004). The results are in good accordance with the reported work of Lal and Gardner, (2012) and Amitsinh V. *et.al.*, 2014. In statistical analysis the paired t-test was done using Minitab 14 software and the experimental model is significant at 95% confidence level because the p-value is < 0.05 that is 0.001.

Optimization of pH: The optimum initial pH of the medium is one of the important factor effecting growth, product formation of microorganisms and the character of their metabolism. The data recorded in figure 6, show that maximum biosynthesis level of tannase by *Streptomyces sp.*SKA1 could be obtained at pH value 7. When the initial pH was 4, the tannase activity was less. As the pH increased, the tannase activity increased and reached a maximum at pH 7. A further increased in pH decreased the tannase activity. This characteristic suggests the neutrophilic nature of the strain which may relate to its natural growing environment. In statistical analysis for pH optimization, calculated p-value is 0.046, as the p-value is less than 0.05 the experiment is in agreement with significance at 95% confidence level.

Optimization of concentrations of agro-residues: Various concentrations of agro-residue, that is leaves of *Azadirachta indica* as a source of tannic acid was used in medium to find out the optimum concentration of agro residue. It was observed that 4% agro residue was suitable for tannase production. In another study, by Sabu A, *et.al.*, 2006.found a mixture of few agro industrial residues like rice bran, sugar cane bagasse etc. in a ratio 1:1 was found to be suitable for tannase production. In statistical analysis for concentration of agro-residue optimization, calculated p-value is 0.049, as the p-value is less than 0.05 the experiment is in agreement with significance at 95% confidence level.

Optimization of carbon source: Cellulose, Tannic acid, Mannitol, Raffinose, Glycerol, Starch, Arabinose, Maltose, Ribose, Xylose, Fructose, Sucrose and glucose) at concentration 1 % were supplemented to the culture medium. Among the sugars tested as carbon and energy source, starch was found to support maximum enzyme production (10.50 U/ml). In statistical analysis for different carbon sources optimization, calculated p-value is 0.042, as the p-value is less than 0.05 the experiment is in agreement with significance at 95% confidence level.

Optimization of Nitrogen source: The effect of nitrogen source on tannase production from *Streptomyces sp.*SKA1 was studied by supplementing the production medium with various organic and inorganic nitrogen sources. The maximum production of tannase was obtained with casein as compared to other organic and inorganic sources. (Figure 9). Arulpandi *et.al.*, 2008 also studied the effect of supplementation of different inorganic and organic nitrogen sources on tannase production. The organic nitrogen sources such as casein and peptone gave considerable enzyme production. In statistical analysis the paired t-test was done using Minitab 14 software and the experimental model is significant at 95% confidence level because the p-value is < 0.05 that is 0.048.

Conclusion

The present work has been taken up with a view of exploring the possibilities of using leaves of *Azadirachta indica* as a substrate and *Streptomyces sp.*SKA1 as a microbial source for the production of tannase which can hydrolyze tannic acid to gallic acid. It makes the process of tannase production economic and ecofriendly, and also suggests a beneficial utilization of agro residues. The present study shows the successful optimization of culture conditions for the maximum tannase production. Optimized culture conditions gave maximum tannase activity (11 U/ml). Based on the results obtained, the culture conditions may be considered for large scale production.

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