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OPTIMISATION OF PARAMETERS FOR ENHANCED TANNASE PRODUCTION FROM A NOVEL BACTERIAL PRODUCER

Sarang Sheela*, Vichare Smita and Vora Dipak

Ramnarain Ruia College, L.N Road, Matunga, Mumbai, Maharashtra, India 400019.

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***Corresponding Author**
Sarang Sheela
Ramnarain Ruia College,
L.N Road, Matunga,
Mumbai, Maharashtra,
India 400019.

ABSTRACT

Tannase (E.C. 3.1.1.20) is an inducible hydrolysable enzyme of great value in various industries. It hydrolyses the ester and depside linkages in hydrolysable tannins to release glucose and gallic acid. Tannase has gained importance in the food, chemical, beverage and pharmaceutical industry as a means of providing cost effective gallic acid. Hence different strategies are required to increase the production of tannase without compromising economic considerations. In this paper, we have reported the isolation and characterisation of a novel bacterial tannase producer. Furthermore, the media components were optimised to achieve high tannase yields using environmentally friendly methods. The effect of nitrogen and carbon sources as well as the effect of tannic acid concentration were studied with a view to obtain high tannase yields. In addition, physical conditions such as the effect of temperatures, pH, aeration and agitation were also investigated. These manipulations have resulted in an overall 1.4 fold increase in tannase production. These studies suggest that the tannase producer under consideration has the potential of being exploited commercially.

KEYWORDS: Tannase, green technology, isolation, media optimisation, high yields.

INTRODUCTION

Tannin acyl hydrolase or tannase is an inducible enzyme (E.C. 3.1.1.20). It hydrolyses the ester and depside linkages in hydrolysable tannins. They release glucose and gallic acid from the hydrolysable tannins. Tannase enzyme has wide ranging uses in various industries. It has found applications in the food, beverage, chemical and pharmaceutical industries. Amongst the important applications of tannase is the production of gallic acid and propyl gallate. The former one is used in the pharmaceutical industry for the synthesis of antibacterial drugs and

in the food industry as substrate for the chemical synthesis of food preservatives such as pyrogallol and gallates such as propyl gallate, a very important food antioxidant. The enzyme has potential to degrade tannery effluents and hence can serve as an environmentally important enzyme. The enzyme is also used as a clarifying and debittering agent of wines, fruit juices and other beverages.

Tannase can be obtained from plants, animals as well as microorganisms .The most important source is microbiological, because the produced enzymes are more stable as compared to those from other sources. Tannins are toxic and bacteriostatic compounds forming irreversible complexes with proteins. They protect the vulnerable parts of the plants from microbial attack by inactivating the invasive microbial extracellular enzymes. Despite the antimicrobial properties of tannins, many fungi, bacteria and yeasts are quite resistant to tannins and can grow and develop on them. It has long been known that several fungal species such as Aspergillus spp. **(Pinto, 2001) (Sabu, 2005)** and Penicillum spp. are capable of producing large amounts of tannase. Over the past decade, many bacterial species have also been reported to produce tannase. These include lactobacilli **(Ayed, 2002) (Sabu, 2006),** *Streptococcus gallolyticus*, *Lactobacillus koalarum* and *Bacillus licheniformis* **(Mondal[a,b], 2000)**.

Due to the difficulties in establishing cost-effective scaling up and downstream processing protocols the large-scale application of tannase is currently limited and expensive. Hence, it is important to find economic ways for the production and purification of tannases.

Soil samples from various locales were collected and screened for the presence of tannase producers. Numerous methods were employed to find a bacterial isolate possessing potent tannase activity. Methods were applied to increase the tannase production via optimisation of media. Further work is conducted to purify tannase enzyme from the bacterial isolate.

MATERIALS AND METHODS

Isolation of tannase producers

Twenty soil samples were used from different areas in Mumbai and Thane region. The soil samples were enriched in nutrient broth for 24 hours followed by isolation on minimal agar plates with 2% tannic acid. The plates incubated at room temperature and observed for growth the next day. Individual colonies from the plates were picked up and streaked on nutrient agar plates to get pure culture. Each of the cultures were then subjected to swarm

plate assay **(Wilson, 2009)**, visual assay **(Osawa, 1993)** and paper chromatography **(Iqbal, 2012)** method to identify a culture with high tannase activity

Identification

From the above tests, it was possible to select the best tannase producer. This culture was identified using standard cultural and biochemical tests. The identity of the organism was further confirmed using 16s rRNA methodology.

Media Optimisation

The culture was then grown in various media to optimise the conditions for tannase production. The basal medium had the following composition (5X):- Ammonium Chloride- 5 g/l; Potassium diphosphate- 15 g/l; Sodium hydrogen phosphate- 33.9 g/l; Sodium Chloride- 2.5 g/l. All the chemicals used were from **SRL (Sisco Research Laboratories)**. After autoclaving, the media was diluted to 1X concentration with sterile distilled water. Further 0.2% 1M sterile glucose and 0.02 % 1M sterile magnesium sulphate and finally Filter sterilised 1% tannic acid was added prior to inoculation of the culture.

Assay of tannase activity was performed according to the method of Sharma et. al**. (2000)**
Media was optimised for various physical conditions such as temperature, static or shaker conditions, media components and their concentrations were also varied to determine the optimum conditions for tannase production by the isolates. All experiments were conducted in triplicates.

RESULTS AND DISCUSSION

Isolation of tannase producers

A total of 70 isolates were obtained during screening. The preliminary test showed isolates which had a zone of clearance around the colonies grown on tannic acid- nutrient agar plates as shown in Figure 1. These colonies were subjected to further tests to eliminate the possibility of selecting false positives. It has been reported that a false halo can be seen because some organisms give out alkaline substances in the medium which is known to show a slight halo **(Osawa & Nishitani, 2005)**.

Fig 1: Zone of clearance around isolate

Hence an array of tests were performed from 24 hours old cultures of the isolates. The results obtained from various tests are as follows.

1. Swarm plate assay: Appropriate swarming was observed indicating that tannic acid was utilised by some of the isolates. The test requires a nutrient gradient to be produced and the chemotaxis of the organism shows that they have grown and used tannic acid for their growth as it is the sole source of carbon in the medium. 20 organisms exhibited swarming effect.
2. Visual detection test: Green to brown coloration of the supernatant indicating tannase production was observed. The tubes with no activity show an orange/yellow coloration, thus showing that tannase was not produced in these organisms. 15 organisms showed green coloration thus indicating that they possessed tannase activity
3. Paper Chromatography: The above two results were confirmed using paper chromatography for the respective isolates. Breakdown product of tannic acid i.e. gallic acid was visualised by all of the 15 isolates.

All these tests, showed that 15 isolates had tannase activity out of which one organism with a relatively high tannase activity was selected.

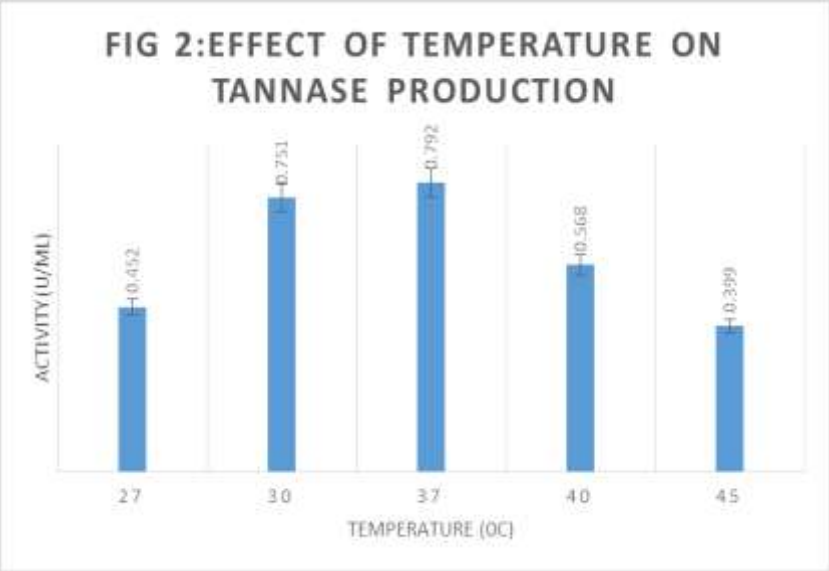
Identification of Culture

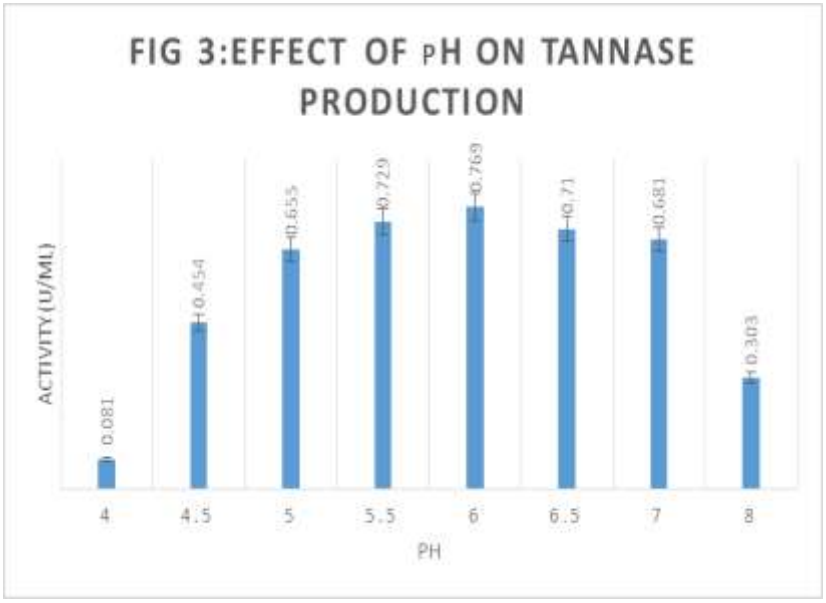
After the tests, the selected organism was subjected to different biochemical tests and 16s rRNA analysis for identification. Using these methods, the selected bacterial isolate was identified as *Serratia marcescens.*

Media Optimisation

It was necessary to carry out the media optimisation to improve upon the tannase production. Different parameters influence the growth of the organism and production of the desired enzyme in the media. These parameters were thoroughly studied.

Temperature and pH: Media was tested with incubation at different temperatures of 27^0C, 30^0C, 37^0C and 40 ^{0}C (Figure 2). *Serratia* showed to have good activity at 37 ^{0}C.Media was also tested at pH 4, 5, 6, 7 and 8 (Figure 3). The pH was adjusted using 1N HCl of 1N NaOH. It is noted that pH 6 induced optimal tannase production.





Static/ Shaker and requirement for oxygen: Tannase production from *Serratia* was tested in anaerobic and aerobic condition as well as on static and shaker conditions for the production of tannase. It showed that the best activity was produced at aerobic static conditions. This shows that while oxygen is required for the growth of the organism (Table 1). Too much aeration can hamper the tannase production (Table 2).

Table 1: Effect of Aeration.

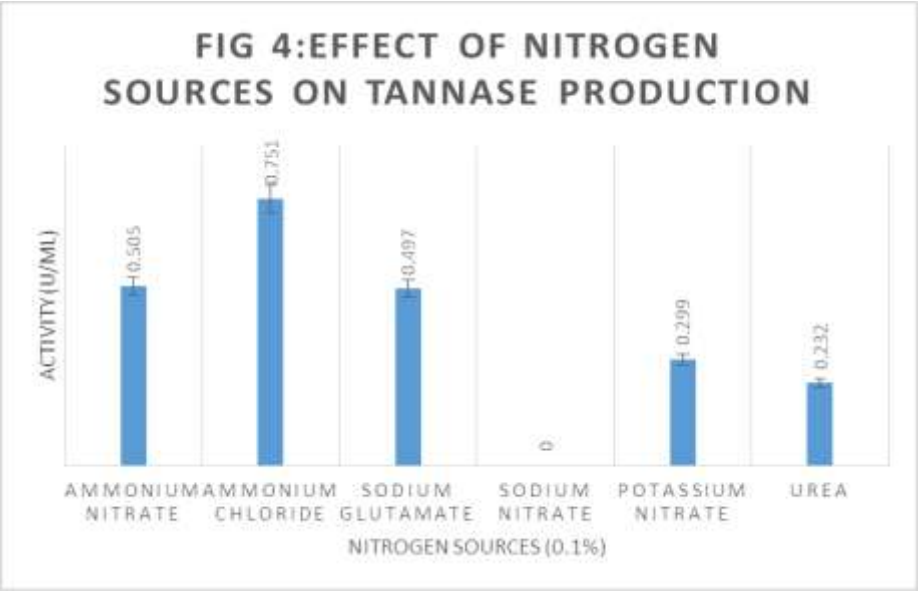
Conditions	Activity (U/mL)
Aerobic	**0.810**
Anaerobic	0.443

Table 2: Effect of Agitation.

Conditions	Activity (U/mL)
Aerobic static	**0.815**
Aerobic shaker	0.694

Effect of media composition on tannase production

Minimal media was used as the base media in which different changes were made so as to increase the tannase production by *Serratia marcescens.* Different nitrogen sources (Figure 4), sugar sources (Figure 6) were used as media components. Sucrose and Ammonium chloride gave best results. Concentrations of ammonium chloride (Figure 5) and sucrose (Figure 7) along with tannic acid (Figure 8) were also varied so as to get best possible combination. It was found that ammonium chloride in 0.1% concentration, sucrose in 0.05% and tannic acid in 1.6% concentration in the media showed optimum production of tannase enzyme.



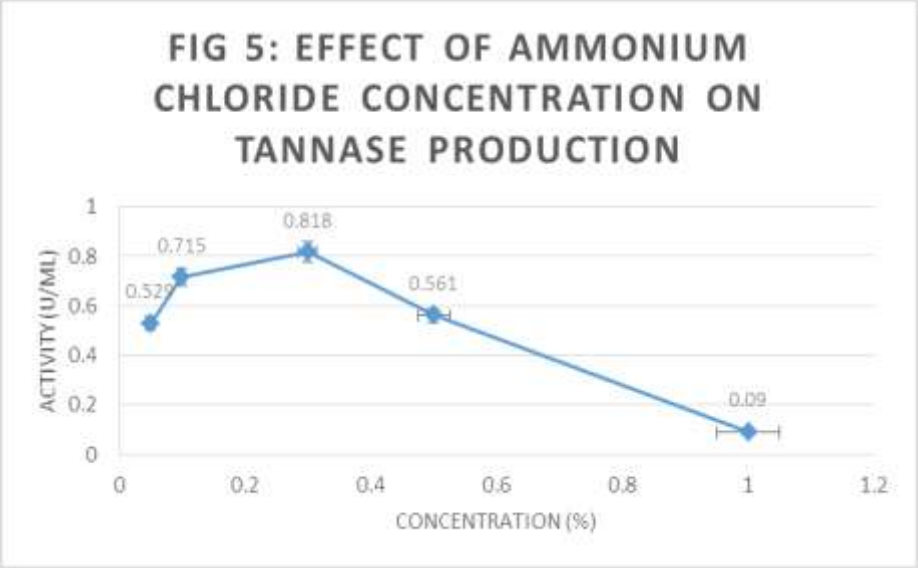


FIG 5: EFFECT OF AMMONIUM CHLORIDE CONCENTRATION ON TANNASE PRODUCTION
ACTIVITY (U/ML)
CONCENTRATION (%)
0.529
0.715
0.818
0.561
0.09
0
0.2
0.4
0.6
0.8
1
1.2

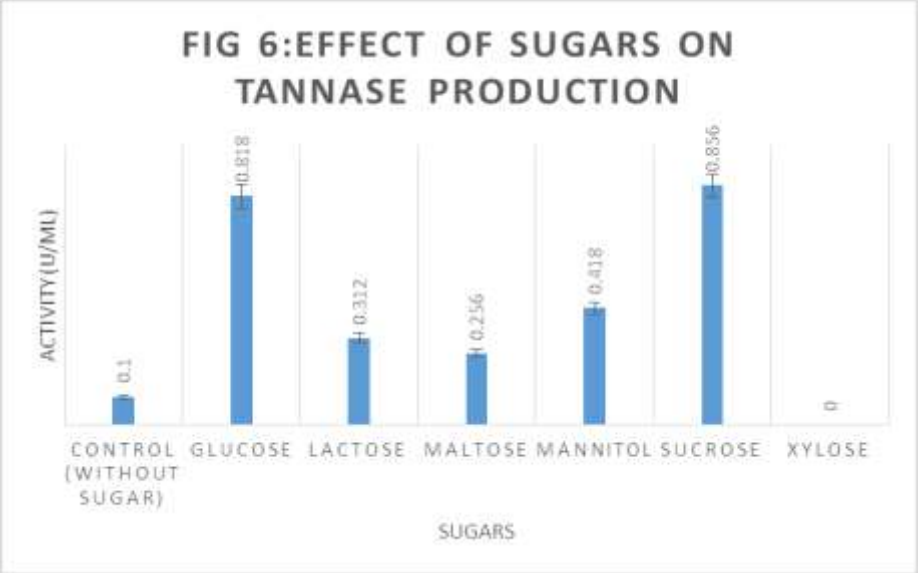


FIG 6:EFFECT OF SUGARS ON TANNASE PRODUCTION
ACTIVITY (U/ML)
SUGARS
0.1
0.818
0.312
0.256
0.418
0.856
0
CONTROL (WITHOUT SUGAR)
GLUCOSE
LACTOSE
MALTOSE
MANNITOL
SUCROSE
XYLOSE

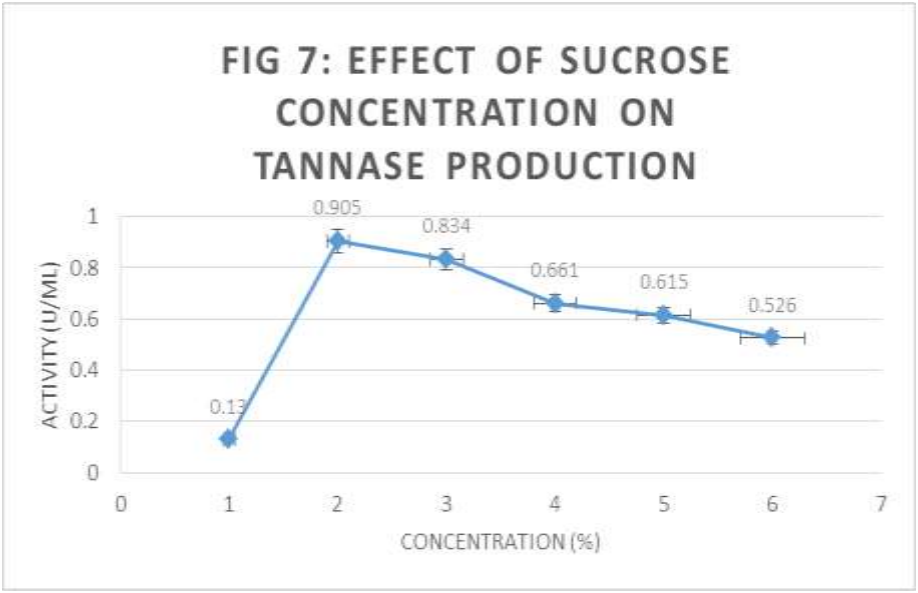
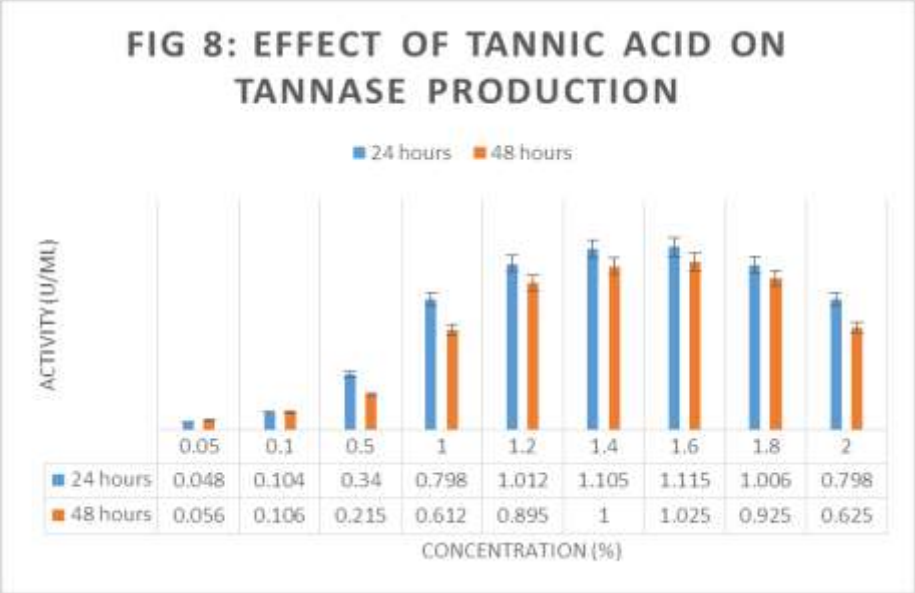


FIG 7: EFFECT OF SUCROSE CONCENTRATION ON TANNASE PRODUCTION
ACTIVITY (U/ML)
CONCENTRATION (%)
0.13
0.905
0.834
0.661
0.615
0.526
0
1
2
3
4
5
6
7



	0.05	0.1	0.5	1	1.2	1.4	1.6	1.8	2
24 hours	0.048	0.104	0.34	0.798	1.012	1.105	1.115	1.006	0.798
48 hours	0.056	0.106	0.215	0.612	0.895	1	1.025	0.925	0.625

CONCLUSION

The tests show that *Serratia marcesans* shows maximum tannase activity at 37^0C aerobic static conditions and using 1.6% tannic acid as well as 0.05% sucrose source. The final activity after all the conditions were finalised was1.115 U/ml. The initial activity found was 0.792 U/ml and through the media optimisation, it was possible to bring about 1.4 fold increase in the activity. The organism shows higher activity than reported by Wilson et. al (2009) for Citrobacter sp isolated from water and soil samples which showed highest activity of 0.5 U/ml after 48 hours. Tannase from Enterobacter cloacae MTCC 9125 (Beniwal, 2010) was optimised by changing conditions and the media components as well. However, it showed maximum activity of 0.6 U/ml after 48 hours of incubation. Beniwal brought about a 1.5 fold increase in the activity of tannase which is comparable to the one achieved in this research paper as well.

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