Tannase Production By *Aspergillus niger*

N. LOKESWARI* and K. JAYA RAJU

Center for Biotechnology,
Department of Chemical Engineering,
Andhra University,
Visakhapatnam- 530003,
Email:lokeswarin@hotmail.com

Received 22 November 2006; Revised 29 December 2006; Accepted 6 January 2007

Abstract: A method for assay of microbial tannase (Tannin acyl hydrolase) based on the formation of chromogen between gallic acid and rhodanine is reported. Maximum Tannase production occurred in the culture broth containing 1-2 % (w/v) tannic acid and 0.05 – 0.1 % (w/v) glucose. The pH, incubation period, temperature and Glucose concentration optima of Tannase production was found at 5.5, 36 h, 35 °C and 0.5% respectively. These properties make the enzyme suitable for pollution control and bioprocess industry. This assay is very simple, reproducible, and very convenient, and with it Tannase activity can be measured in relation to the growth of the organism. *Aspergillus niger* exhibited higher enzyme activity showing about 65 mole percent conversion respectively after a 36 h incubation period. The assay is complete in a short time, very convenient and reproducible.

Keywords: Tannic acid, Gallic acid, Gallo tannin, Tannase, Tannin.

Introduction

Tannin acyl hydrolase commonly called tannase is produced by a number of microorganisms like fungi (*Aspergillus*, *Penicillium*, *Rhizopus* sp), yeast (*Candida* sp), and bacteria (*Bacillus* sp)¹⁻². The major commercial application of this enzyme is in the hydrolysis of gallotannin to Gallic acid, is an intermediate required for the synthesis of an antifolic antibacterial drug trimethoprim³. Tannase is extensively used in the preparation of instant tea, wine, beer, and coffee – flavored soft drinks and also as additive for detannification of food⁴. Purification and evaluation of the enzyme require a sensitive, reproducible, and convenient assay method. A number of chromogenic methods have been described for the assay of tannase that are not specific⁵. Madhavakrishan *et al.* reported a method for tannase assay based on the estimation of glucose liberated by incubation with the enzyme for 24 h, which is not suitable for routine assays of the enzyme⁶. Iibuchi *et al.* described a spectrophotometric method that has been used by many workers in its original and modified forms¹⁻².

This method was based on the decrease in absorbance of the substrate Tannic acid at 310 nm. Some workers have assayed tannase by measuring gallic acid using chromatographic techniques such as gas chromatography⁷, or high – performance liquid chromatography⁸. But each method has its own limitations and most are time consuming and also require instrument sophistication. Considering this, Inoue and Hagerman, have described a method for the determination of gallotannins, which involves the formation of a chromogen between gallic acid obtained by the acid hydrolysis of gallotannins and rhodanine⁹. This was later adapted by¹⁰, for the assay of tannase in a ruminal bacterium. It is simple, reproducible, and with it tannase activity can be measured in relation to the growth of the organism.

The present paper, reports the production of tannase from a newly isolated *Aspergillus niger* in liquid culture medium by submerged fermentation technique. Some properties of tannase have also been studied.

Experimental

Chemicals

Gallotannin was bought from National Chemicals, Nutan, Gujarat, India. Tannic Acid was bought from Loba chemical Pvt. Ltd. Bombay, India. Potato sucrose agar from Himedia. Acetic acid, sodium acetate, ferric chloride, hydrochloric acid, potassium hydroxide were bought from Loba chemical Pvt. Ltd, Bomba, India. Triethanolamine, sodiumdodecyl sulphate, sulphuric acid were bought from Ranbaxy. Bovineserum albumin was bought from Sigma.

Microorganism

Aspergillus niger obtained from Institute Microbial Technology, Chandigarh was used for the present work. *Aspergillus niger* grows on Czapek yeast extract agar medium containing 0.01 % gallotannin. It grows rapidly at room temperature (25 °C) and at 37 °C. The slant cultures were then used for further work or stored in refrigerator at 4 °C.

Preparation of Spore Suspension:

8 mL of sterile distilled water was taken in 50 mL conical flask. The mycelia of the slant cultures were scraped off in 2 mL of distilled water. The resulting spore suspension was mixed to obtain a uniform suspension. This suspension was then added to distilled water to give 10 mL of spore suspension for spore dilution.

Preparation of induced inoculum

Tannase being an adaptive enzyme, preinduced inoculum is required to be prepared. The medium used for growing fungi, *Aspergillus niger* was potato dextrose broth containing 0.5% gallotannin adjusted to pH 5.6. 50 mL gallotannin containing potato dextrose medium was taken, then each one is transferred to respective 250 mL conical flask and then sterilized. These flasks were inoculated aseptically with 2 mL of spore suspension prepared from the culture slants. These flasks were kept in a rotatory shaker (160 rpm) at 32 °C for 48 h. After 48 h of incubation this induced inoculum was used for subsequent studies of tannase production.

Fermentation method for enzyme production

Enzyme productin was carried out in 250-mL Erlenmeyer flasks containing 50-mL 2% tannic acid in potato dextrose broth (pH 5.5) medium was taken in 100 mL conical flasks. Then it

was sterilized and inoculated with 2% (v/v) inoculum was added and incubated at 35 °C for 36 h in a rotary shaker (200-rpm). The culture supernatants obtained by filtration (through Whatman No.1 paper) were assayed periodically for tannase activity. Enzyme production has also been carried out in presence of different concentrations of glucose, incubation period, pH, and Temperature. Growth of the organism was estimated on the basis of biomass dry weight (mg/mL).

Partial purification of tannase

Enzyme obtained from the culture filtrate and homogenized mycelia were precipitated with solid ammonium sulfate (60-90% saturation) at 4 °C for overnight. The precipitate was collected by centrifugation (15,000 x g, 15 min), dissolved in citrate buffer (0.1 M, pH 5.0) and dialyzed against the same buffer for 2 days. The dialysates were used as the source of partially purified enzyme for the study of enzyme properties.

Assay of tannase

Tannase activity was determined by the method of Mondal and Pati¹¹. Enzyme solution (0.1 mL) was incubated with 0.3 mL of 1.0% (w/v) tannic acid, in 0.2 M acetate buffer (pH 5.0) at 40 °C for 30 min and then the reaction was terminated at 0 °C by the addition of 2 mL BSA (1 mg/mL), which precipitates the remaining tannic acid. A control reaction was also done side by side with heat denatured enzyme. The tubes were then centrifuged (5,000 x g, 10 min) and the precipitate was dissolved in 2 mL of SDS – triethanolamine (1% w/v, triethanolamine) solution and the absorbency was measured at 550 nm after addition of 1 mL of FeCl₃ (0.13 M) .

One unit of the tannase was defined as the amount of enzyme, which is able to hydrolyse 1μ mole of ester linkage of tannic acid in 1 min at specific condition.

Results and Discussion

In the present work, studies on the tannase production from *Aspergillus niger* was carried out and the results were given in the Tables and Figs. The effect of some parameters at different ranges was studied and their influence on the production was discussed in this paper.

Optimization of some parameters for maximum Tannase production

1. Effect of glucose conc. (% w/v) on tannase production

To study the effect of different glucose conc. on tannase production, the glucose concentration of the medium was varied from 0.01 % to 1% (w/v). It has been found that glucose at higher concentration repressed tannase synthesis while the lower concentration is not repressive. Maximum enzyme production occurred at 0.05% (w/v) glucose, respectively in Tannic acid medium. Higher concentration of glucose repressed enzyme production due to the availability of ready made carbon source maximum tannase activity was 21.42 U/mL by *Aspergillus niger* after the optimum glucose concentration of 0.5 % (w/v). The obtained results were tabulated in Table 1 and also shown in Fig 1. Earlier Beverini *et al.* also mentioned that lower concentration of glucose is not repressive for enzyme production in *A. Japonicas* but its concentration above 1.0% is inhibitory for both growth and enzyme production¹².

Table 1. Effect of glucose concentration % (w/v) on tannase production

S.No.	Glucose conc. (%, w/v)	Tannase activity (U / mL)
1.	0.01	16.61
2.	0.05	19.22
3.	0.1	20.32
4.	0.5	21.42
5.	1	18.32
6.	1.5	17.24

Figure 1. Effect of glucose concentration on tannase production

2. Effect incubation period (h) on tannase production

To evaluate the effect of different incubation period on tannase production, the incubation period of the medium range was varied from 12 h to 72 h. With a rise in incubation period, the tannase production increased and optimum activity was recorded at 36 h (Fig. 2). With a further increase in incubation period, there was a decrease in activity. Maximum tannase activity was 20.26U/mL by the *Aspergillus niger* after the optimum incubation period of 36 h. The obtained results were tabulated in Table 2 and also shown in Fig 2. An optimum incubation period around 36 h has been reported for tannase activity in case of *A. aculeate* D B F 9¹³.

Table 2. Effect of incubation period on tannase production

S.No.	Incubation period, (h)	Tannase activity (U / mL)
1.	12	12.62
2.	24	16.38
3.	36	20.26
4.	48	18.42
5.	60	14.83
6.	72	13.42

3. Effect of pH on tannase production

To study the effect of initial pH on Tannase production, the pH of the medium was varied from 3.5 – 6.0 using 1N HCl and 1N NaOH, and fermentation was done as usual. The enzyme was active at acidic pH and activity decreased as the pH approached the alkaline range. The optimum tannase production was recorded at pH 5.5 (Fig.3). Maximum tannase activity was 22.62 U/mL by the *Aspergillus niger* after the optimum pH of 5.5. Any change in pH affects the protein structure and a decline in enzyme in activation or its instability. It could be concluded from the results that tannase from the *Aspergillus niger* needed an acidic environment to be active. Fungal tannase is an acidic protein in general. The obtained results were tabulated in Table 3. and also shown in Fig 3. There are reports describing of the optimum pH as 5.5 in case of tannase obtained from *A. oryzae*¹⁴ and 6.0 in case of tannase obtained from *P.chrysogenum* and *A. niger*¹⁵.

Table 3. Effect of pH on tannase production

S.No	pH	Enzyme activity (U/mL)
1	3.5	14.32
2	4	16.28
3	4.5	18.64
4	5	20.02
5	5.5	22.62
6	6	16.42

Figure 3. Effect of pH on tannase production

4. Effect of temperature on tannase production

To study the effect of different temperatures on tannase production, the flasks containing medium kept at temperature range was varied from 25 – 50 °C. With a rise in temperature, the tannase production increased and optimum activity 23.52U/mL was recorded at 35 °C (Fig.4). With a further increase in temperature, there was a decrease in activity. The optimum temperature for tannase production was 35 °C. The obtained results were tabulated in Table 4 and also shown in Fig 4. An optimum temperature around 30 °C has been reported for tannase activity in case of *A. oryzae*¹⁶ and *P.chrysogenum*, around 35 °C in case of *A.niger* and 50 °C in case of *candida* sp.¹⁷.

Table 4. Effect of temperature on tannase production

S.No.	Temperature (°C)	Tannase activity U / mL
1.	25	12.20
2.	30	16.52
3.	35	23.52
4.	40	18.48
5.	45	16.22
6.	50	15.43

Figure 4. Effect of Temperature on tannase production

Conclusions

The present work has been taken up with a view of exploring the possibilities of using *Aspergillus niger* as a source for the production of tannase which can hydrolyse gallotannin to gallic acid. The high yields of tannase (24.26 U/mL) under optimum conditions show that the method is quite efficient and the tannase producing fungi *Aspergillus niger* gave high yield of tannase activity 65.8%. This strain is able to produce tannase in the medium containing Tannic acid as the sole carbon source.

References

1. Iibuchi S, Minoda Y, and Yamada K, *Agric. Biol. Chem*, 1967, **32**, 513 – 518.
2. Rajakumar G.S, and Nandy S.C, *Appl. Enviorn. Microbiol*, 1983, **46**, 525 – 527.
3. Sitting M, In Pharmaceutical Manufacturing Encyclopedia, 2nd (eds.). 1988, 282-.
4. Lekha P K, and Lonsane B K, *Chem Microbiol; Technol Lebensm*, 1993, **44**, 215.
5. Lekha P K, and Lonsane B K, *State of the art. Adv. Appl. Microbiol*, 1997, **44**, 215.
6. Madhavakrishna W, Bose S M, and Nayudamma Y, 1960, *Bull. CLRI* **7**, 1-11.
7. Jean D, Pourrat H, Pourrat A, and Carnat A, *Anal. Biochem*, 1981, **110**, 369-372.
8. Niehaus J U, and Gross G G, *Phytochemistry*, 1997, **45**, 1555 – 1560.
9. Inoue K H, and Hagerman A E, *Anal. Biochem*, 1988, **169**, 363 - 369
10. Skene I K, and Brooker J D *Anaerobe*, 1995, **1**, 321-327.
11. Mondal K C, and Pati B R, *J. Basic Microbiol*, 2000, **40**, 223-232
12. Beverini M, and Metche M, *Sci Aliments* 1990, **10**, 807-16.
13. Lekha P K, and Lonsane, B K, *Process Biochem*, 1994, **29**, 497-503.
14. Yamada H, Adachi M, Watanabe M, and Sato N, *Agric Biol Chem*, 1968, **32**(9), 1070.
15. Barthomeuf C, Regerat F, and Pourrat H, *J. Ferment Bioeng*, 1994, **77**(3), 320-3.
16. Ibuchi S, Minoda Y, and Yamada K, *Agric Biol chem*. 1968, **32**(7), 803- 809.
17. Aoki K, Shinke R, Nishira H, *Agric Biol Chem* 1976, **40**(1), 79-85.