International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.4, No.4, pp 1461-1467, Oct-Dec 2012

Optimization of Submerged Fermentative Production of Tannase by Aspergillus flavus

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Abstract: A study on optimization of submerged fermentative production of tannase by *Aspergillus flavus* was carried out. *Aspergillus flavus* was selected out of three fungal isolates have the ability to grow on the presence of tannic acid. Tannase enzyme production by Submerged Fermentation (SmF) from the agro-industrial residues namely Redgram Husk and Tamarind seed powder as substrates was studied using *Aspergillus flavus* (MTCC-3783).For *Aspergillus flavus,* the optimum Tannic Acid concentration and fermentation conditions were found to be 3 %, pH 5.5, temperature 35°C and fermentation period of 96 h.
Key words: Submerged fermentation, Tannase, Agro residue, Aspergillus flavus.

1. Introduction

Tannin acyl hydrolase commonly known as tannase, catalyses the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid, resulting glucose and gallic acid [1].The enzyme is extensively used in the preparation of instant tea, beer, wine, coffee flavored soft drinks and also additive for detannification of food. A potential use of tannase is in the treatment of waste water contamination with polyphenolic compounds such as tannic acid [2].Tannase is used in the production of gallic acid, a substrate for chemical synthesis of propyl gallate and trimethoprim which have application in the food and pharmaceutical industries [3,4]. Fungi cultures are mainly used for tannase production [5,6] and some yeast[7] and bacteria[8] also could be used. In Industrial level tannase is mainly produced by Aspergillus species under submerged fermentation (SmF).The SmF is widely used for enzyme production because it offers many advantages like uniform process conditions namely concentration, Temperature, pH, aeration and agitation in the bioreactors [1]. Many authors reported the tannase production by *Aspergillus* species in the medium containing pure tannic acid as both inducer and carbon source. Agro residues and forest products are generally considered as the best sources of tannin rich substrates for SmF [9].
In the present research work, the tannase enzyme production was studied by Submerged fermentation technique using *A.flavus* and Tamarind seed powder and Red gram husk as substrates. The classical method was used for optimizing the process conditions.

2.Materials And Methods

2.1 Substrate Preparation

Powdered Agrowastes were used for the production of Tannase enzymes. The Agrowastes Greengram Husk, Blackgram Husk, Tamarind Seed powder, Redgram Husk, Tea dust, Rice bran and Groundnut shell were powdered to 100 mesh (0.15 mm) fine powders using laboratory grinder

at 3000 rpm and was preserved in a sealed plastic bag at 4°C to prevent any possible degradation or spoilage.

2.2 Microorganism and Culture conditions

The tannase producing fungal cultures namely *Aspergillus flavus* (MTCC 3783), *Aspergillus foetidus* (MTCC 3557) and *Aspergillus niger* (MTCC 282) were obtained from IMTECH, Chandigarh and were used for tannase production. All the three fungal cultures were maintained on Czapek Dox minimal media agar slants supplemented with 1 % tannic acid as the sole carbon source. The fungal strains were sub cultured periodically, grown at 30ºC for 7 days. The well grown cultures were stored at 4°C in a refrigerator and used for further subculturing.

2.3 Screening of Microorganisms for the production of Tannase by Plate Assay Method

Screening was performed in plates of selected medium for *Aspergillus flavus* (MTCC 3783), *Aspergillus foetidus* (MTCC 3557) and *Aspergillus niger* (MTCC 282). The composition of the Czapek Dox minimal medium used for tannase enzyme production was Tannic Acid 10 g/L, Sodium nitrate - 6 g/L, Potassium dihydrogen orthophosphate- 1.52 g/L, Magnesium sulphate- 0.52 g/L, Potassium chloride- 0.52 g/L, Ferrous sulphate- 0.01 g/L, Zinc sulphate- 0.01 g/L and Agar- 30g/L. The fungal culture was inoculated on the center of the plates and incubated at 30ºC for 72 hours. The diameters of the growth were measured at different incubation periods namely 24, 48, 72 h and 96 h respectively. *A. flavus* was found to grow well when compared to *A. foetidus* and *A. niger* and is shown in Fig.1, Fig.2 and Fig.3.*A.flavus* was selected for further studies on optimization of process conditions for maximum production of tannase enzyme.

2.4 Production of Tannase in submerged fermentation (SmF)

The spore suspension was inoculated in 250 ml Erlenmeyer flask containing 100 ml of Czapek Dox minimal medium.3 gm of substrates (mentioned in Section 2.1) were added separately to the above mentioned medium for studying their effect on the enzyme production. The cultures were grown at 30°C, 140 rpm for six days in an incubator shaker. The samples were withdrawn at regular intervals of 24 h. The biomass was separated by the filtration through Whatman No.1 filter paper. The cell free culture broth was assayed for the tannase activity.

2.5 Assay of tannase (Mondal and Pati) [10]

0.1 ml of enzyme solution was incubated with 0.3 ml of 1.0% (w/v) tannic acid and 0.2 M acetate buffer (pH 5.0) at 40 °C for 10 min and then the enzyme production was stopped by cooling to 0°C by the addition of 2 ml Bovine Serum Albumin (BSA) (1 mg/ml), which precipitates the remaining tannic acid simultaneously A control without the enzyme was incubated and the samples were analyzed. The tubes were then centrifuged (5,000 x g, 10 min) and the precipitate was dissolved in 2 ml of Sodium Dodecyl Sulphate (SDS) – triethanolamine (1% w/v SDS in 5% v/v triethanolamine) solution and the absorbency was measured at 550 nm after addition of 1 ml of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01N HCl).

One Unit of the tannase enzyme was defined as the amount of enzyme required to hydrolyse 1µ mole of ester linkage of tannic acid in 1 min at specific condition.

Fig. 1. Production of tannase using *A. flavus*

Fig. 2 Production of Tannase using *A. foetidus*

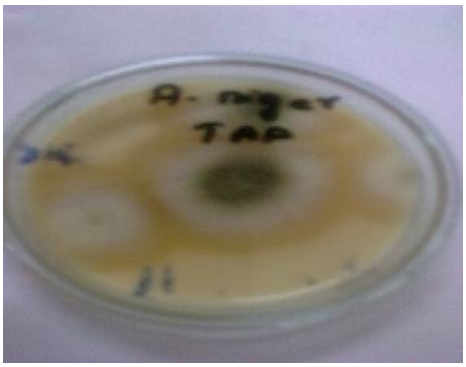

Fig. 3 Production of Tannase *A. niger*

3. Results And Discussion

The selection of substrates for a large scale enzyme production by fermentation depends on its easy availability, cost and production efficiency. Several low cost agro residues were used for effective, economical production of tannase by *A.flavus* through SmF and the best substrate for tannase production was selected. Various parameters were optimized to obtain maximum tannase production.

3.1Effect of Substrate on production of Tannase

The effect of substrate on tannase enzyme activity in submerged fermentation using *Aspergillus flavus* was studied by conducting experiments with different substrates namely Tamarind seed powder (TSP), Redgram Husk (RGH), Greengram Husk (GGH), Blackgram Husk (BGH), Groundnutshell (GNS), Teadust (TD) and Rice bran (RB). The flasks containing Czapek Dox minimal media were incubated with 3% (w/v) of the above mentioned substrates in a rotary shaker at 35^0C, pH 5.5 and 200 rpm. The samples were drawn at regular time intervals of 24 h and analyzed for tannase activity at 48h, 72h, 96h, 120h and 144h. The results are given in Table 1 and Fig.4 for tannase activity obtained at different timings with various substrates. The substrates studied Redgram Husk and Tamarind seed powder gave the maximum tannase activity of 76.49 U/ml and 67.59 U/ml at a fermentation period of 96 h. This higher production with RGH and TSP may be due to the higher tannin content and the substrates are easily metabolizable by *A.flavus*. The other substrates namely GNS, RB, BGH, GGH and TD gave a maximum tannase production of 66.12 U/ml, 39.67 U/ml, 35.26 U/ml, 30.81 U/ml, and 24.05 U/ml respectively at 96 h. After 96 h of fermentation, the enzyme production was found to decrease because of the non- availability of the substrates. Since maximum tannase production was obtained with RGH and TSP, both the substrates were selected for further studies on tannase enzyme production for 96h.Sabu et al reported that 96h of fermentation period gave the maximum tannase production with palm kernel cake as substrate. [11]

Table 1. Effect of substrate on Production of Tannase using *A. flavus*

	Tannase Activity (U/ml)						
Time (h)	BGH	GGH	RGH	RB	GNS	TSP	TD
0	0	0	0	0	0	0	0
24	1.40	1.10	5.89	1.98	3.65	6.30	6.24
48	2.33	3.45	12.22	3.44	5.58	10.11	11.14
72	8.81	14.96	47.02	17.69	23.51	35.26	17.04
96	35.26	30.81	76.49	39.67	66.12	67.59	24.05
120	23.57	22.24	51.43	21.57	24.98	47.02	22.04
144	14.69	14.98	24.1	21.74	22.04	29.39	14.4

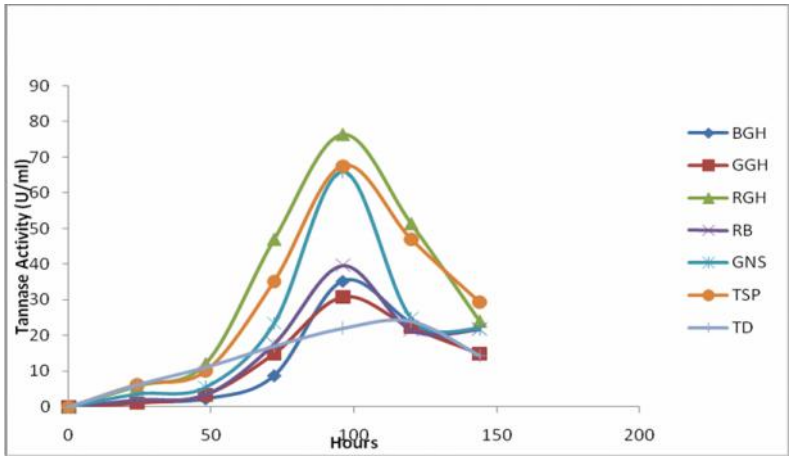

Fig.4. Effect of Substrate on Tannase Activity using *A.flavus.*

3.2 Effect of Temperature on production of Tannase

The effect of temperature on tannase enzyme production was studied by conducting experiments at different temperatures namely 25°C, 30°C, 35°C, 40°C and 45°C keeping other parameters constant at pH 5.5, 200 rpm with the substrate concentration of 3% .The results are given in Table 2 and Fig 5. As temperature was increased the maximum tannase enzyme production was found to increase rapidly and was similar to the "effect of temperature on chemical reactions". The maximum enzyme production obtained were 20.57 U/ml, 51.43 U/ml, 76.44 U/ml, 61.72 U/ml and 54.37 U/ml at 25°C, 30°C, 35°C, 40°C and 45°C respectively with RGH as a substrate. The maximum enzyme production obtained with TSP as a substrate were 44.89 U/ml, 55.84 U/ml, 67.63 U/ml, 48.49 U/ml and 24.98 U/ml at 25°C, 30°C, 35°C, 40°C and 45°C respectively. At low temperatures 25°C and 30°C the rate of enzyme production was found to be less and the maximum enzyme production was found to be less. At high temperature of 40°C and 45°C the growth rate of *A.flavus* was found to be less and this phenomena is similar to catalyst deactivation or catalyst poisoning in chemical catalysts. The enzyme probably got denatured which in turn resulted in less enzyme production. The optimum temperature was 30°C for various fungi such as *A.japonicus* and *A.aculeatus* [12, 13] *A.oryzae* [14] and *A.ruber* [15]. The maximum tannase enzyme production of 76.44 U/ml and 67.63 U/ml were obtained at 35°C and were chosen as the optimum temperature for RGH and TSP as substrates respectively. The optimum temperature 35°C was used for further studies.

3.3 Effect of pH on production of Tannase

The effect of pH on tannase enzyme production was studied by conducting experiments at different pH namely 4.5, 5, 5.5, 6 and 6.5 keeping other parameters constant at temperature 35°C, 200 rpm with the substrate concentration of 3% .The results are given in Table 3 and Fig 6.As pH was increased the tannase enzyme production was found to increase. The maximum tannase enzyme production obtained were 20.57 U/ml, 32.33 U/ml, 76.41 U/ml, 47.03 U/ml and 23.51 U/ml at pH 4.5, 5, 5.5, 6 and 6.5 respectively with RGH as a substrate. The maximum enzyme production obtained with TSP as a substrate were 35.26 U/ml, 38.20 U/ml, 67.58 U/ml, 45.55 U/ml and 36.73 U/ml at pH 4.5, 5, 5.5, 6 and 6.5 respectively. The maximum tannase enzyme production of 76.41 U/ml and 67.58 U/ml were obtained at pH 5.5 and were chosen as the optimum pH for RGH and TSP as substrates respectively. The optimum pH 5.5 was used for further studies. In the maximum tannase enzyme production was found at pH 5.5.This may be due to the preference of fungi cultures for acidic medium. The optimum pH for tannase production has been found to vary from 4.5 to 6.5 in different fungi [16] and bacteria [17, 18]

Table 2. Effect of Temperature on Production of Tannase using *A.flavus*

Temperature (C)	Tannase Activity (U/ml)	
	RGH	TSP
25	20.57	44.89
30	51.43	55.84
35	76.44	67.63
40	61.72	48.49
45	54.37	24.98

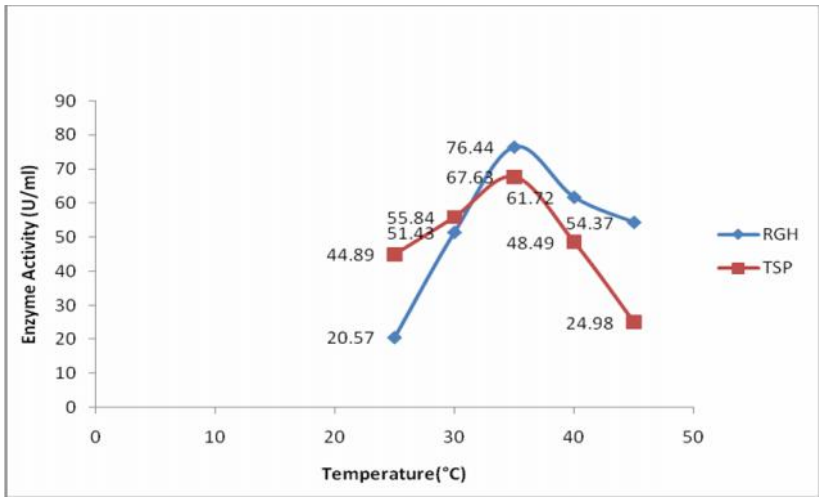

Fig.5. Effect of Temperature on Production of Tannase using *A.flavus*

Table 3. Effect of pH on Production of Tannase using *A.flavus*

pH	Tannase Activity (U/ml)	
	RGH	TSP
4.5	20.57	35.26
5.0	32.33	38.20
5.5	76.41	67.58
6.0	47.03	45.55
6.5	23.51	36.73

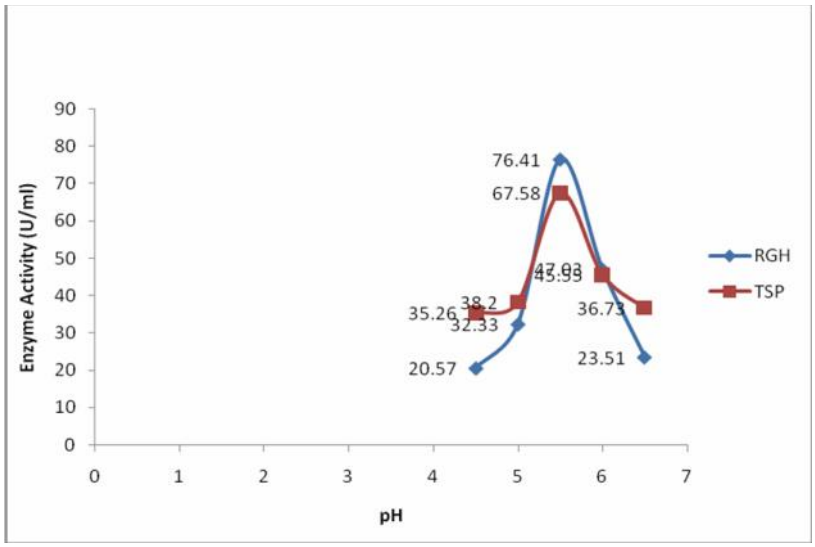

Fig 6 Effect of pH on Production of Tannase using *A.flavus*

3.4 Effect of concentration of Tannic acid on production of Tannase

The effect of concentration of tannic acid on tannase enzyme production was studied by conducting experiments at different concentrations namely 1%, 2%, 3%, 4% and 5% keeping other parameters constant at temperature 35°C, pH 5.5 and 200 rpm. The results are given in Table 4 and Fig 7. As the tannic acid concentration was increased the tannase enzyme production was found to increase. The maximum enzyme production obtained were 22.04 U/ml, 46.75 U/ml, 76.43 U/ml, 44.50 U/ml and 28.04 U/ml at tannic acid concentration of 1%, 2%, 3%, 4% and 5% respectively with RGH as a substrate. The maximum enzyme production obtained were 19.60 U/ml, 39.60 U/ml, 67.58 U/ml, 36.50 U/ml and 22.44 U/ml at tannic acid concentration of 1%, 2%, 3%, 4% and 5% respectively with TSP as a substrate. The maximum tannase enzyme production of 76.43 U/ml and 67.58 U/ml were obtained with RGH and TSP as a substrates respectively at tannic acid concentration of 3% (w/v) and was chosen as the optimum tannic acid concentration for. The optimum tannic acid concentration 3% was used for further studies. The enzyme production was found to decrease at higher inducer concentration of 4% and 5% and may be due to the inhibition of inducer. Similarly Selwal M.K et al observed an inhibitory effect due to higher concentration of tannic acid in the tannase synthesis by *P.Aeruginosa* IIIB 8914 [19] using agro residues like wheat bran, palm kernel cake, amla and keekar leaves.

Table 4. Effect of Tannic Acid concentration on Production of Tannase using *A.flavus*

Tannic acid Concentration (%)	Tannase Activity (U/ml)	
	RGH	TSP
1	22.04	19.60
2	46.75	39.60
3	76.43	67.58
4	44.50	36.50
5	28.04	22.44

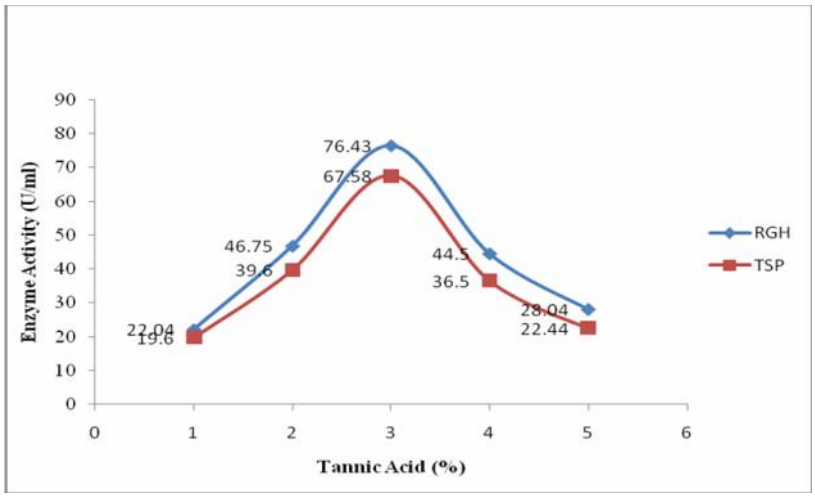

Fig 7 Effect of Tannic Acid concentration on Production of Tannase using *A.flavus*

Conclusion

The present investigation suggests that agro residues such as RGH and TSP are one of the best and most cost effective alternatives for the costly pure tannic acid for industrial production of microbial tannase. The tannase produced by *A.flavus* has interesting characteristics and this fact encourages further studies including its production at industrial scale. Tannin acyl hydrolase is an industrially important enzyme that is mainly used in the food and pharmaceutical industry. As the range of application of this enzyme is very wide there is always a scope for novel tannase with better characteristics, which may be suitable in the diverse field of applications.

Acknowledgments

The authors gratefully acknowledge the Bioprocess research Laboratory, Department of Chemical Engineering, Annamalai University for providing the necessary facilities for the sucessful completion of this research work.

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