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Enhanced Tannase Production Using *Aspergillus Flavus* by Fed-Batch Fermentation with Redgram Husk

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ABSTRACT

Tannin acyl hydrolase also known as tannase (EC 3.1.1.20) is a hydrolytic enzyme that catalyses the hydrolysis of (hydrolysable) tannins such as tannic acid, resulting glucose and gallic acid. The enzyme has wide applications in food, beverage, brewing and chemical industries. It is mainly used for the preparation of Gallic acid, instant tea, wine, coffee flavored soft drinks, clarification of beer and fruit juices. Enhancement of tannase production is required to meet its current demand. The aim of the present study is to enhance the production of tannase enzyme using *Aspergillus flavus* (MTCC 3783) using Redgram Husk as substrate in a 3L fermenter by batch and fed-batch fermentation. By applying a fed-batch strategy, production of tannase could be almost doubled as compared to Batch fermentation using the substrate Redgram Husk with the yield of 160.14 U/ml and 88.46 U/ml respectively. The maximum production of tannase using Redgram Husk showed the suitability of this culture process for industrial-scale development.

Keywords: Tannase, Fed batch fermentation, Redgram Husk, *Aspergillus flavus*

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INTRODUCTION

Tannase (tannin acyl hydrolase, E.C 3.1.1.20) is an extracellular hydrolytic enzyme that catalyses the hydrolysis of ester and depside bond in hydolysable tannins or gallic acid esters, liberating glucose and gallic acid as a final product[1]. Tannase enzyme has been used extensively in the preparation of instant tea, beer, wine, coffee flavored soft drinks and also additive for detannification of food. 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 [2, 3]. Fungi cultures are mainly used for tannase production [4, 5] and some yeast[6] and bacteria[7] also could be used. In Industrial level tannase is mainly produced by Aspergillus species under submerged fermentation (SmF). Fed-batch culture is an efficient technique to control the organism's growth rate and achieve high yields of biomass and metabolites by avoiding the repressive effects of high substrate concentration. The above-mentioned approach has been successfully applied in the productions of tannase by yeast *Arxula adeninivorans* [8]. Many Authors reported the optimization and production of tannase by batch fermentation; however, there are limited papers describing tannase production by fed-batch fermentation. The aim of the present study was to develop a fed-batch process for enhanced production of tannase by *Aspergillus flavus* using redgram husk as a novel substrate.

MATERIALS AND METHOD

Chemicals

Chemicals used in the experiments were purchased from Hi-Media, Mumbai and were of the highest purity.

Strain

The fungal strain *Aspergillus flavus* (MTCC 3783) for tannase production obtained from IMTECH, Chandigarh, India was maintained in Modified Czapek Dox agar media with a composition of sucrose 30 g/l, sodium nitrate 2 g/l, magnesium glycero phosphate 0.50 g/l, potassium chloride 0.50 g/l, dipotassium sulphate 0.35 g/l, ferrous sulphate 0.01 g/l and agar 12 g/l at 30ºC for 7 days.

Inoculum preparation

The inoculum required for submerged fermentation is prepared using Modified Czapek Dox medium harvesting the spores from 96 hours old culture (grown at 35ºC). The spore suspension was collected in sterile falcon tube and stored at 4ºC until the actual study.

Fermentation conditions

Erlenmeyer cultivation

The Erlenmeyer flasks containing culture, Modified Czapek Dox Media supplemented with 3% (w/v) of the substrate (redgram husk) were incubated in a rotary shaker at 35°C, pH at 5.5, 200 rpm for a fermentation period of 144 hours. The samples were drawn at regular time intervals of 12 hours and analyzed for tannase activity.

Batch and Fed-batch cultures

A modular fermenter (New Brunswick, USA) of 3L capacity was used to study the kinetics of tannase enzyme production. The fermenter was equipped with automatic control of agitation, temperature, pH, dissolved oxygen concentration and foaming. The Modified Czapek Dox Medium of 1L and 3% (w/v) of substrate redgram husk was sterilized for 15 minutes at 121°C and was cooled after the sterilization. 50 ml of the seed culture of *A.flavus* was used to inoculate the sterile medium. The production of tannase was carried out under optimum operating conditions at 35^{o}C, an initial pH of 5.5 and with an inducer tannic acid concentration of 3% (w/v) for a fermentation period of 120 hours. The samples were drawn at regular time intervals and analyzed for tannase activity and biomass concentration. The fed-batch culture was started with a batch growth phase, followed by a feeding phase. 3% (w/v) of substrate redgram husk was fed continuously into the fermenter at a rate of 9 ml / hr. The fermentation was carried out under optimum operating conditions of 35^{o}C, an initial pH of 5.5 and with an inducer tannic acid concentration of 3% (w/v). The fermentation was stopped at 108 hours since there is no change in the biomass concentration and enzyme concentration. The samples were drawn at regular time intervals and analyzed for tannase activity.

Assay of Tannase

0.05 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 3 ml Bovine Serum Albumin (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 (5000 x g, 10 min) and the precipitate was dissolved in 3 ml of Sodium Dodecyl Sulphate - triethanolamine (1% (w/v) SDS in 5 % (v/v) triethanolamine) solution and the absorbency was measured at 530 nm after addition of 1 ml of $FeCl_3$ (0.01 M $FeCl_3$ in 0.01 N HCl). One Unit of Tannase activity is defined as the amount of enzyme required to hydrolyze one micro mole of ester linkage of tannic acid in 1 min at specific condition[9].

Biomass Estimation

A known volume of fermented broth was taken and centrifuged. Then the supernatant separated out from the fermented broth for analyzing the activity and the settled cell mass was dried and weighed. The difference between initial and final weight of centrifuge tube will be the weight of the cell mass[10].

RESULTS AND DISCUSSION

Tannase Production in Shake Flask Culture

The media with substrate redgram husk gave the maximum tannase activity of 70.49 U/ml at a fermentation period of 96 hours. After 96 hours of fermentation, the enzyme production was found to decrease because of the non- availability of the substrates. This higher tannase production with redgram husk as substrate may be due to the higher tannin content and the substrates are easily metabolizable by *A.flavus*. Sabu et al reported that 96 hours of fermentation period gave the maximum tannase production with palm kernel cake as substrate[11]. The results are shown in Figure.1.

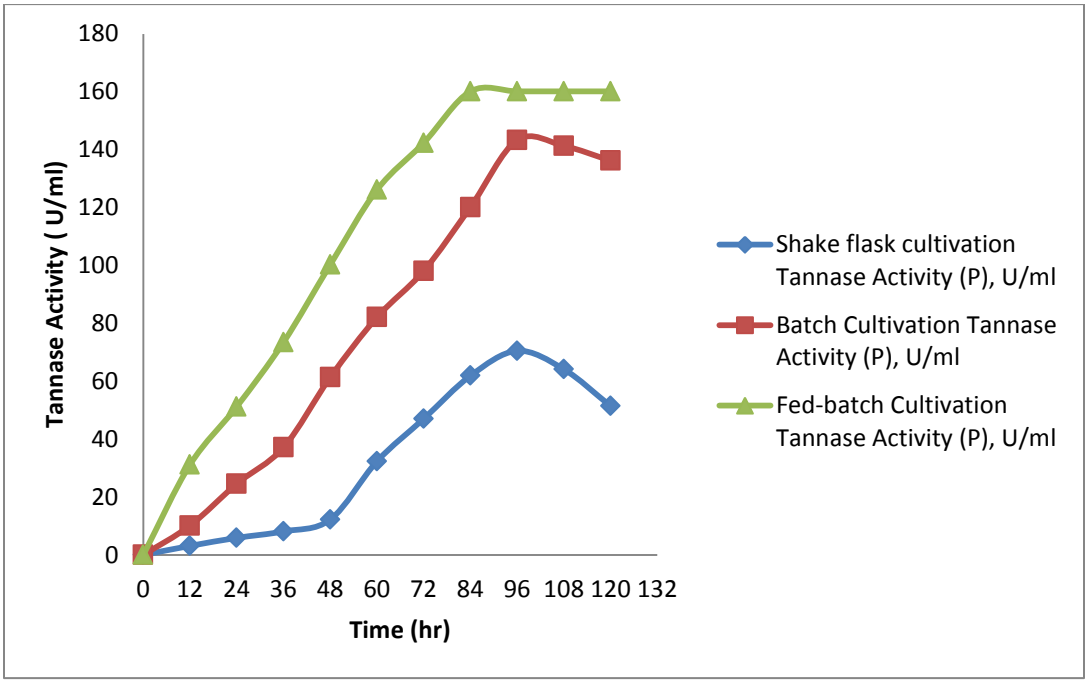

Figure 1: Effect of Time on Tannase enzyme Production by shake flask, batch and Fed-batch cultivation using *A.flavus* with RGH as substrate under optimum conditions.

Tannase Production in Batch Culture

The tannase batch production data presented in Figure 2 revealed that substrate redgram husk support *A. flavus* growth and enzyme production. The tannase activity was found to increase with respect to fermentation time and reaches a maximum of 143.30 U/ml at the end of 96 hours of

fermentation and then decreases gradually till the end of fermentation. The biomass concentration reaches a maximum of 6.62 g/l at 96 hours and further there was no increase in biomass concentration till the end of the fermentation. These values were twofold higher than those obtained using shake flask culture. The significant increase of tannase enzyme production by batch fermentation compared to the shake flask culture could be explained by the improved oxygen transfer in the bioreactor. Oxygen limitation could be a serious problem in the shaken flask cultivations due to the highly non-Newtonian medium that is caused by the filamentous growth of the fungus[12].

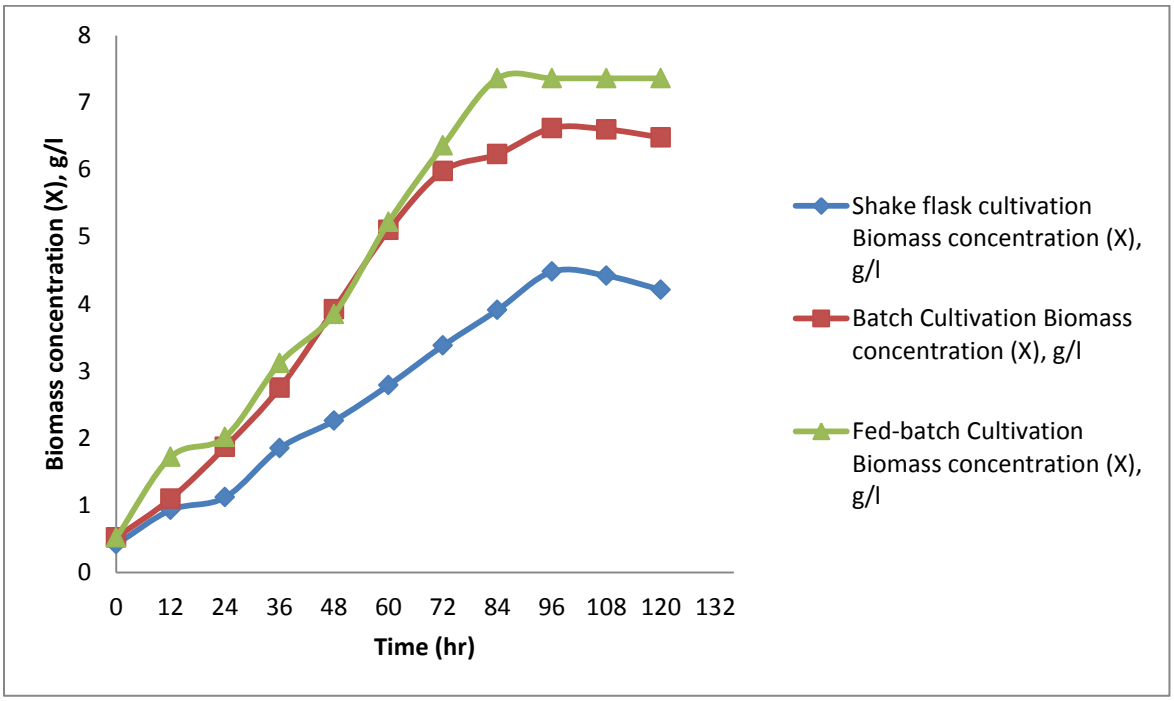

Figure 2: Effect of Time on Biomass concentration by shake flask, batch and Fed-batch cultivation using *A. flavus* with RGH as substrate under optimum conditions.

Tannase Production in Fed-Batch Culture

Figure.1 and Figure.2 show that the tannase enzyme activity and biomass concentration are found to increase exponentially from 0 to 84 hours and reaches a maximum tannase activity of 160.14 U/ml and a maximum biomass concentration of 7.36 g/l at the end of the 84 hours fermentation (Table.1). This simultaneous increase in tannase activity with biomass concentration is due to the growth associated nature of the product tannase. Further the biomass concentration and tannase enzyme activity remain constant till the end of the fermentation period of 108 hours. This result may be explained by the accumulation of the substrate which had an inhibitory effect on fungal growth and tannase production.

Table 1: Comparison of Tannase Activity and Biomass concentration by shake flask, batch and Fed-batch cultivation using *A.flavus* with RGH as substrate.

Run No	Time (hr)	Shake flask cultivation		Batch cultivation		Fed-batch cultivation	
		Biomass concentration (X), g/l	Tannase Activity (P), U/ml	Biomass concentration (X), g/l	Tannase Activity (P), U/ml	Biomass concentration (X), g/l	Tannase Activity (P), U/ml
1	0	0.42	0	0.52	0.00	0.52	0
2	12	0.93	3.12	1.10	10.14	1.72	31.20
3	24	1.12	5.89	1.87	24.59	2.02	51.23
4	36	1.85	8.12	2.75	37.14	3.12	73.45
5	48	2.26	12.22	3.92	61.36	3.85	100.25
6	60	2.79	32.33	5.10	82.12	5.22	126.12
7	72	3.38	47.02	5.98	98.12	6.36	142.34
8	84	3.91	61.89	6.23	120.14	7.36	160.14
9	96	4.48	70.49	6.62	143.30	7.36	160.14
10	108	4.42	64.12	6.60	141.30	7.36	160.14
11	120	4.21	51.43	6.48	136.21	7.36	160.14

CONCLUSION

The results obtained in the present study indicate high tannase production by culturing *A.flavus* in fed-batch using redgram husk as substrate. The highest tannase activity reached 160.14 U/mL in the fed-batch culture using redgram husk as substrate. So redgram husk could be used as a less expensive substrate for an efficient tannase production at industrial scale.

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