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Evaluation of Feeding Strategies for Enhanced Cell-Associated Tannase Production by Serratia Ficaria DTC

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Abstract: Batch studies on Cell-associated tannase production showed 2.6 U/L activity in the declining phase of growth in the bioreactor. It was observed that Cell-associated tannase production under declining phase was depending upon the bacterial biomass produced under exponential phase and gallic acid level. The peak production of enzyme was always accompanied by a sharp rise in dissolved oxygen concentration. Based on these observations, fed batch fermentation by feeding a mixture of nutrients (glucose and tryptose) and Dissolved oxygen (DO) based feeding strategy of gallic acid were designed. Nutrient feeding strategy showed 10 U/L of enzyme activity at 14^{th} h of fermentation. DO based feeding strategy of gallic acid resulted in the production of 14.4 U/L enzyme activity in the 12^{th} h of fermentation. The enzyme production rate of 1.2 U/L.h achieved in this mode was 4.6–fold greater than the values observed in batch process and 1.68 fold greater than the productivity achieved by feeding nutrients. Hence, DO based feeding strategy of gallic acid was proved to be an effective strategy for enhanced cell-associated tannase production by Serratia ficaria DTC.

Keywords: *Cell-associated tannase, Fed-batch process, Serratia ficaria*

Introduction:

Tannin acyl hydrolase (E.C. 3.1.1.20), commonly known as "Tannase" catalyses the hydrolysis of ester and depside bonds in hydrolysable tannins. Hydrolysis of gallotannins (Tannic acid) results in the release of gallic acid and glucose. Tannase finds use in the production of gallic acid, which is used for the manufacture of an antimalarial drug, Trimethoprim and an antioxidant, propyl gallate. Tannase is being used for the production of instant tea preparations, wines, juices of fruits and refreshing drink with coffee flavor (Belmares et al. 2004). Tannase has potential uses in the treatment of tannery effluents and pre-treatment of tannin containing animal feed (Lekha and Lonsane 1997). Many molds, yeasts and bacteria have the ability to produce tannase. Majority of the reports are pertaining to extracellular tannase production from bacteria and fungi (Aguilar et al. 2007). For the first time, Belur et al. (2010) had reported cell-associated tannase (CAT) activity in several bacterial isolates. CAT provides a unique naturally immobilized form of tannase. Such naturally immobilized tannases have many advantages as they avoid expensive and laborious operation of isolation, purification and immobilization. Further more, natural immobilization has higher recovery and yield compared with chemical or physical immobilization. Naturally immobilized enzymes invariably shows very high stability against adverse pH and temperature compared to the free enzyme (Kopecny and John Wallace 1982, Sinsuwan et al. 2008). Culture strategy greatly affects the effectiveness of a process. All the available literature on tannase production made use of batch processes. It has inherent disadvantages such as substrate limitation, catabolite repression and feedback inhibition. Fed-batch culture mode emerges consequently as an attractive choice in numerous biotechnological processes due to its operational simplicity, reliability and flexibility for the implementation in multipurpose facilities (Gordillo et al. 1998) In the present paper, two different fed-

batch strategies, Dissolved Oxygen (DO) based feeding strategy of gallic acid and nutrient feeding strategy were evaluated for enhanced tannase production.

Materials and Methods:

Microorganism and Culture Conditions:

Serratia ficaria DTC (MTCC 8930) capable of producing CAT, isolated previously in our laboratory was used for the current study (Belur et al. 2010). The culture was maintained on nutrient agar slants at 4ºC.

Analytical Methods:

Tannase activity was measured by determining the amount of gallic acid released by hydrolysis of tannic acid. Since tannase produced by Serratia ficaria DTC is cell associated, bacterial biomass obtained by centrifugation at 5500 x g for 15 min was washed with buffer and used as source of enzyme. Tannase activity was determined as per the method described by Van de Lagemaat and Pyle (2001). One unit of tannase activity is defined as the amount of enzyme required to release 1 µmole of gallic acid per minute under standard reaction conditions. Glucose content of fermentation broth was determined by 3,5 dinitro salicylic acid method (DNS method) using D-glucose as standard. Gallic acid content of fermentation broth was determined using gallic acid as standard as per the method described by Van de Lagemaat and Pyle (2001). Growth was monitored by measuring dry cell weight (DCW). DCW was determined by centrifuging the fermentation broth at 5500 x g, 15 min, and sediments were dried at 95°C for 36 hours. All the estimations were made in triplicate and mean values were considered for the analysis.

Bioreactor Studies:

Batch fermentations were carried out in 3 L stirred tank bioreactor (Scigenics India) containing 1.7 L medium. Medium used for bioreactor studies had glucose 20 g/L, tryptose broth (Himedia India) 30 g/L. Gallic acid (4 g/L) was used as the inducer in the place of tannic acid. Poly propylene glycol 0.2 %(v/v) was used as the antifoam. 12 h old culture, cultivated in 250 ml Erlenmeyer flask containing 100 ml of nutrient broth (13 g/L) was used as the inoculum. 5% v/v of inoculum was used. The reactor was maintained at an aeration of 5 LPM, agitation of 800 rpm at 30ºC. Sterilizable DO and pH probes (Mettler Toledo) were used for monitoring. pH of the process was maintained at 5 through out, by adding appropriate amount of 1 M NaOH and HCl. Fed-batch experiments were conducted in the same bioreactor with the same operating conditions as mentioned above. The process was started with medium consisting of glucose (20 g/L), tryptose broth (30 g/L) and gallic acid (4 g/L). Glucose and tryptose mixture was fed in the form of pulses at 7^{th} hr and 7.5^{th} hr which incidentally corresponds to the declining phase of growth. Each pulse of 10 ml had the composition of glucose 2.125 g, tryptose 1.175 g which was based on substrate utilization rate under batch conditions. DO based fed-batch experiment was started with medium consisting of only glucose (20 g/L) and tryptose broth (30 g/L). Sharp rise in DO due to decreased growth rate during the transition of exponential phase to stationary phase was used as an indicator to give gallic acid pulse. 20 ml of solution containing 6.8 g gallic acid neutralized with 1 M NaOH was used as gallic acid pulse. Batch and fed-batch trials were conducted in duplicate and mean values were taken for analysis. pH of the process was maintained at 5 by adding appropriate amount of 1 M NaOH and HCl during the fermentation.

Results and Discussion:

Batch Cultivation in Bioreactor:

Most of the reports pertaining to the microbial production of tannase have reported tannic acid as an inducer for tannase production. Gallic acid was proved to be an alternative inducer to tannic acid by same authors earlier (unpublished). Hence gallic acid 4 g/L was used as an inducer in present studies. Fig 1 shows the profiles of DCW, DO (% saturation) and CAT activity of Serratia ficaria DTC under batch cultivation. Exponential phase terminated approximately at 6 h and was followed by declining phase

from 6 to 12 h (transition phase between exponential and stationary phase) and subsequently culture entered stationary phase. Enzyme production was minimal under exponential phase and reached 2.6 U/L under declining phase (10 h) indicating non-growth associated characteristics of tannase production. Reduced growth rate during declining phase was accompanied by steady rise in DO (% saturation) and onset of stationary phase was indicated by sharp rise in DO from 45 to 65 %. Growth parameters of batch cultivation are given in Table 1. Analysis of residual glucose concentration strengthens the logic that reduction in growth rate was due to nutrient depletion. Simultaneous consumption of glucose and gallic acid as carbon sources was also noticed. Perhaps, depletion of gallic acid in the broth by the time declining phase was reached could be the reason for low tannase activity. It was evident from results that CAT production under declining phase was directly proportional to the bacterial biomass produced under exponential phase and gallic acid levels during declining phase. Based on these conclusions following two strategies were evaluated for enhanced CAT production.

1. Fed batch fermentation using mixture of glucose and tryptose.
2. DO based feeding strategy of gallic acid.

Fed-Batch Fermentation with Nutrient Feeding:

Nutrient mixture consists of gucose and tryptose was fed in the form of pulses which prolongs exponential phase and thereby increases biomass concentration. It was based on logic that enhanced bacterial biomass under declining phase in the presence of gallic acid expresses higher CAT. Fig. 2 shows the profile of DO (% saturation), cell concentration (DCW), CAT activity, residual glucose and gallic acid level under fed-batch cultivation. Glucose and tryptose mixture was fed in the form of pulses at 7^{th} hr and 7.5^{th} hr in order to prolong the exponential phase which terminated at 6^{th} hr in batch process with similar medium and conditions. Each pulse of 10 ml had the composition of glucose 2.125 g, tryptose 1.175 g based on substrate utilization rate under batch conditions. Addition of glucose and tryptose mixture prolonged exponential phase by 4hrs and shifted maximum tannase production by 4hrs. Specific growth rate and biomass concentration at maximum tannase activity was increased to 0.465 h^{-1} ,12 g/L (14^{th} hr) in fed-batch strategy compared to 0.303 h^{-1},11.52 g/L (10^{th} hr) in batch process respectively. Fed-batch fermentation using mixture of glucose and tryptose produced CAT activity of 10 U/L at 14^{th} hr. Fed-batch production of CAT by Serratia ficaria DTC enhanced tannase production by 3.8-fold, compared to batch production conducted with the same medium and conditions. Reasons for enhanced production of cell-associated tannase in fed-batch fermentation using mixture of glucose and tryptose are higher biomass level during maximum tannase production phase (declining phase) and higher level of gallic acid under declining phase when bacteria is under stress, looking for alternative carbon sources. Sharp rise in DO from 40 to 65% at 13^{th} h indicating onset of stationary phase was in agreement with limitation of glucose in fermentation broth.

Do Based Feeding Strategy of Gallic Acid:

Fig. 3 shows the profile of DO (% saturation), cell concentration (DCW) and residual glucose level under fed-batch cultivation. Sharp rise in DO level from 25 % to 51% at 10^{th} h was used as an indicator of onset of stationary phase to feed gallic acid pulse of 20 ml. Sharp rise in DO was in agreement with limitation of glucose in fermentation broth. Gallic acid pulse addition induced maximum CAT activity of 14.4 U/L at 12^{th} h and consequently reduced due to reduction in cell concentration. Fed-batch process enhanced tannase production by 5.5-fold, compared to batch production conducted with the same medium and conditions. Growth parameters and tannase production in batch and fed-batch fermentation are compared in Table 1. Fed-batch process is a very promising culture mode for the production of secreted proteins

with regard to the maximization of volumetric productivity. Numerous reports are available on utilizing fed-batch strategy for maximizing production. But most of the applications involve the evasion of feedback inhibition / feedback repression or catabolite repression by feeding the carbon or nitrogen sources (Singh et al. 2004; Turki et al. 2010). Some reports are available where fed-batch strategy was used to maintain DO level at a favorable range so as to achieve the desired productivity (Barberis and Sagovia 1997; Kole et al. 1998). But reports on using fed-batch strategy for inducing the enzyme synthesis by feeding the inducer are scarce. Hofer et al. (2002) had successfully demonstrated the use of this strategy to induce Acetopyruvate hydrolase production by Psuedomonas putida, by feeding the inducer Orcinol. Effective and robust fed-batch control strategies are required for the success of fed-batch process. Development of these strategies requires model-based control and on-line monitoring of process variables closely related to cell metabolism. However, these strategies normally involve relatively complicated control models requiring online estimation of cell density, specific growth rate and cellular yields. Monitoring the control variables other than pH and DO is often difficult and requires specialized sensors and analytical instruments (Nor et al. 2001. Reports on DO based feeding strategies in fed-batch process are in plenty. However, reports on DO based induction strategy are few in number. Use of DO based induction strategy for tannase production does not require any real time data regarding specific growth rate, substrate utilization rate or complex control system. Simple DO Probe which gives real time data regarding DO (% saturation) of fermentation broth under constant aeration and agitation could be used effectively to enhance tannase production.

Conclusion:

- The experimental work thus reiterates that fed-batch fermentation is superior to batch fermentation in case of enzyme production.
- DO based feeding of gallic acid was found to be more productive compared to nutrient feeding strategy.
- Probably this is the first report of feeding the inducer making use of novel and simple DO based feeding strategy, producing an inducible enzyme.

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Table 1: *Characteristics of Cell-Associated Tannase Production from Serratia Ficaria DTC in Batch and Fed-Batch Fermentation*

Parameters	Batch process	DO based Fed- batch process	Fed- batch with nutrient feed
Maximum apparent growth rate μ (h^{-1})	0.303	0.37	0.465
Maximum biomass production (g/L)	17	8.77	12.2
Maximum biomass productivity (g/L.h)	0.684	0.49	0.76
Total glucose utilization (g/L)	18	8	14.82
Average glucose utilization (g/L.h)	1.125	0.5	0.988
Maximum tannase production (U/L)	2.6	14.4	10
Average tannase productivity (U/L.h)	0.26	1.2	0.714
$Y_{p/glucose}$ (U/g)	0.144	1.8	0.674

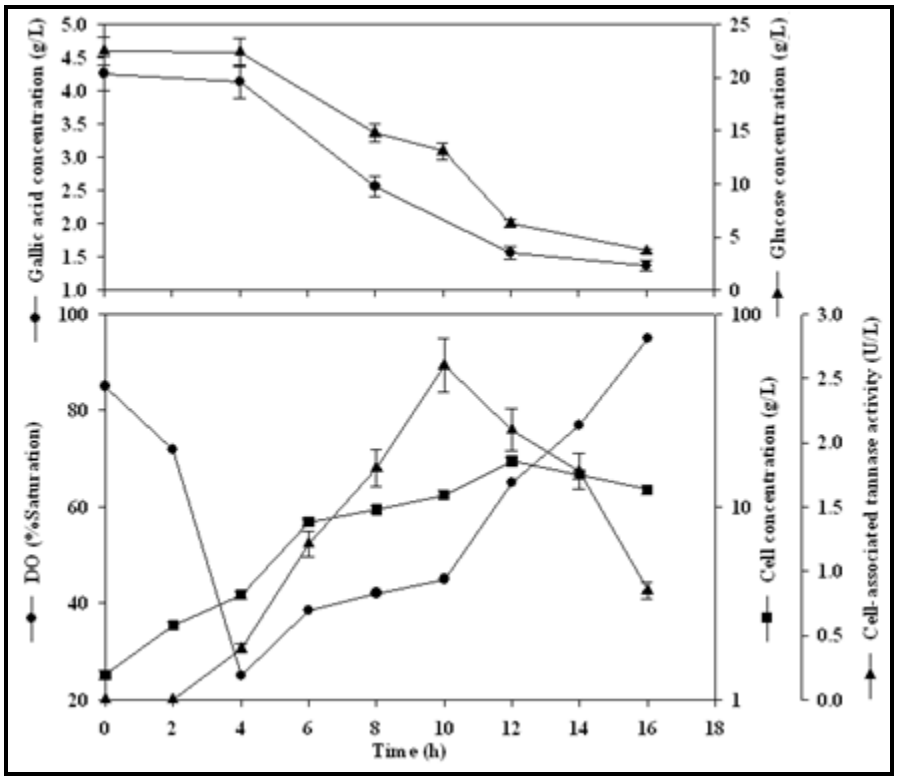

Figure 1: *Batch Cultivation of Serratia Ficaria DTC in MEDIUM HAVING GLUCOSE (20 g/L), Tryptose Broth (30 g/L) and Gallic Acid (4 g/L) in Stirred Tank Bioreactor at Process pH 5.0. (a) Profile of Glucose Concentration and Gallic Acid Concentration (b) Profile of DO (% saturation), Cell Concentration (DCW) and Cell-Associated Tannase Activity.*

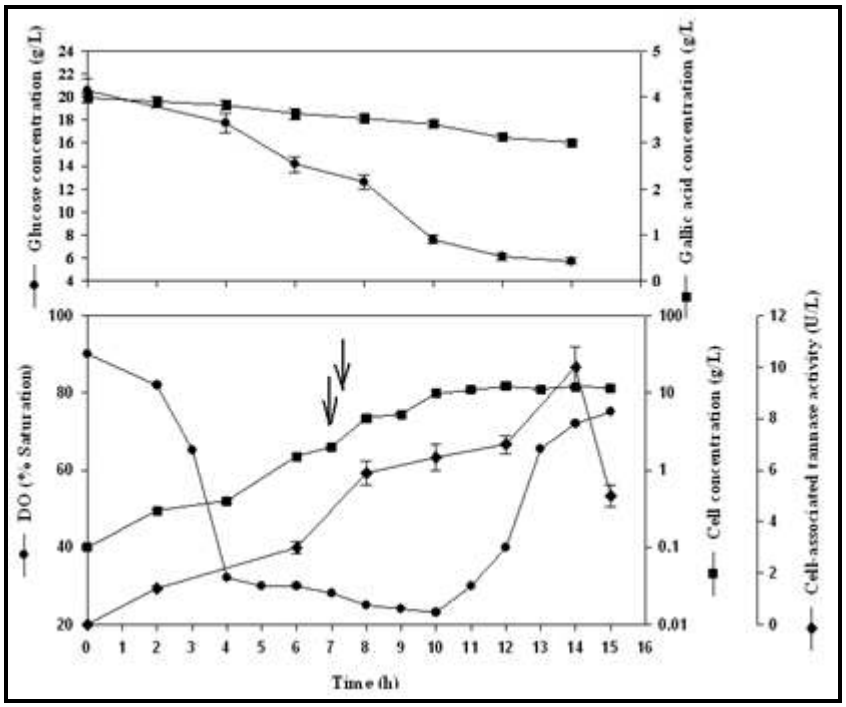

Figure 2: *Profile of Cell Concentration (DCW), DO (% Saturation), Glucose Concentration and Cell-Associated Tannase Activity during Fed-Batch Cultivation of Serratia Ficaria DTC. Medium Consists of Glucose (20 g/L) and Tryptose Broth (30 g/L) and Gallic Acid (4g/L). 10 ml Nutrient (Glucose 2.125 g and Tryptose 1.175 g Mixture) was Fed at 7th and 7.5th h.*

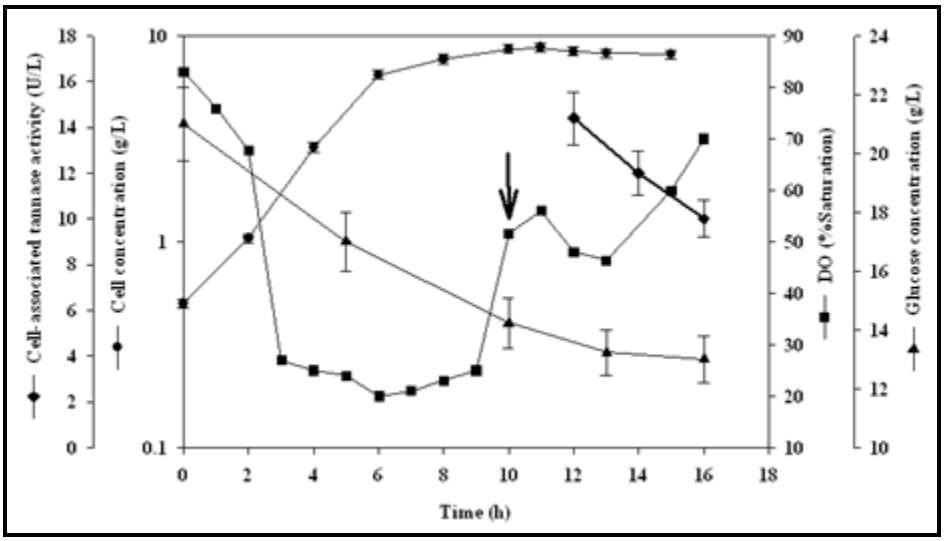

Figure 3: *Profile of Cell Concentration (DCW), DO (% Saturation), Glucose Concentration and Cell-Associated Tannase Activity during Fed-Batch Cultivation of Serratia Ficaria DTC. Medium Consists of Glucose (20 g/L) and Tryptose Broth (30 g/L). 20 ml Gallic Acid Solution (34 % w/v) was Fed as a Pulse at the 10th h when DO was Sharply Increasing.*