

Gallotannin hydrolysis by immobilized fungal mycelia in a packed bed bioreactor

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Hydrolysis of gallotannin to gallic acid by immobilized mycelia of *Aspergillus niger* MTCC 282, *Aspergillus fischerii* MTCC 150, *Fusarium solani* MTCC 350 and *Trichoderma viride* MTCC 167 in a packed bed bioreactor was studied. Fungal mycelia preinduced with 5 g L⁻¹ gallotannin were immobilized in calcium alginate gel (1.5%) and the resultant beads were packed in a column to a bed volume of 175 mm³. Gallotannin dissolved in distilled water was passed through the column and the eluate was recycled after adjusting pH to 6 with ammonium hydroxide (10%). Maximum hydrolysis of gallotannin was recorded by immobilized mycelia of *F. solani* and *T. viride* at 35° and 45°C after 175 and 60 min of residency period respectively. Optimum substrate concentration required for maximum hydrolysis was 10 g L⁻¹ at pH 5 for both the fungi. Immobilized mycelia of *A. niger* and *A. fischerii* revealed maximum operational stability. Loss of activity after eighth run was in the order of – *A. niger* (no loss), *A. fischerii* (7.5%), *F. solani* (18%) and *T. viride* (18%). Stability in terms of retention of enzyme activity after 150 days of storage at 4°C was *A. niger* (58%), *A. fischerii* (26.8%), *F. solani* (83%) and *T. viride* (85.1%).

Microbial production of tannin acyl hydrolase (EC 3.1.1.20), commonly referred as tannase, is well documented¹⁻⁴. Major commercial application of tannase include hydrolysis of gallotannin to gallic acid, a key intermediate required for manufacture of an antifolic antibacterial drug trimethoprim⁵, manufacture of instant tea⁶ and clarification of beer, wine and fruit juices⁷. Growing cells of bacteria and fungal mycelia have been frequently employed for bioconversion of gallotannin to gallic acid⁸⁻¹¹. Although, tannase entrapped in reverse micelle system¹² and adsorbed on silica¹³ have been used for the esterification of gallic acid to propyl gallate, the reports on gallic acid production using immobilized fungal mycelia are scanty¹⁴.

Calcium alginate is commonly used gel matrix for entrapment immobilization of enzymes and microbial cells due to mild conditions required during gelification, low diffusional barrier for molecules below the molecular weight 5000 Dalton¹⁵ and low cost. Looking to the advantages associated with immobilized preparations in catalyzing the biochemical reactions, the present work was undertaken to assess the feasibility of

calcium alginate entrapped fungal mycelia for gallotannin to gallic acid conversion in a packed bed bioreactor. The present communication reports the effect of some parameters on this bioconversion by calcium alginate entrapped mycelia of *Aspergillus niger* (MTCC 282), a fungus commonly used for tannase production and *Aspergillus fischerii* (MTCC 150), *Fusarium solani* (MTCC 350) and *Trichoderma viride* (MTCC 167), the fungi recently reported to produce tannase enzyme when induced with gallotannin present in the incubation medium¹⁶.

Organisms and chemicals—The fungi used in the present study were procured from Microbial Type Culture Collection, Chandigarh, India. Stock cultures were grown on 0.01% gallotannin supplemented potato dextrose agar slants and maintained at 4°C. Sodium alginate was purchased from Sigma Chemical Company, USA and gallotannin was gifted by Kisalaya Pharmaceuticals Ltd., Pithampur, India.

Growth conditions and immobilization—*F. solani* and *T. viride* were grown in potato dextrose broth containing gallotannin (5 g L⁻¹) adjusted to pH 5.6. *A. fischerii* was grown in mineral salt medium containing (g L⁻¹) - sucrose,

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30.0; NH_4NO_3 , 1.65; KNO_3 , 1.90; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.37; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.44; KH_2PO_4 , 0.17; gallo-tannin, 5.0 and (mg L^{-1}) H_3BO_3 , 6.20; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 16.90; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 8.60; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.025; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.5; Na_2EDTA , 7.6; pH adjusted to 5.6. Fungi were incubated on a gyratory incubator shaker (160 rpm, $30^\circ \pm 2^\circ\text{C}$) for 48 hr, the mycelia were collected by filtration, washed with distilled water and blotted in filter paper folds. Calcium alginate beads were prepared according to the method described by Kierstan and Bucke¹⁵ with slight modification. Sodium alginate slurry (1.5%) was stocked with 5% (w/v) mycelia and the mixture was extruded through a nozzle into chilled 0.2 M calcium chloride solution and cured in it overnight. The cured beads were washed with distilled water and stored in 0.02 M calcium chloride solution at 4°C . The spores of *A. niger* were raised on wheat bran moistened with mineral salt solution for ten days at $30^\circ \pm 2^\circ\text{C}$ and 80% relative humidity. The spores were harvested, immobilized in calcium alginate (0.5% loading, w/v) and incubated on a shaker (160 rpm) in 5 g L^{-1} gallotannin supplemented potato dextrose broth at $30^\circ \pm 2^\circ\text{C}$ for 48 hr to obtain mycelia entrapped in calcium alginate beads. Average diameter of the beads was (mm), *A. fischerii*, 3.0; *F. solani*, 3.5, *T. viride*, 3.0 and *A. niger*, 2.1.

Packed bed bioreactor—A 500 mm long glass column (23 mm inner diameter) was packed with mycelia entrapped alginate beads in 70 ml of gallotannin solution of desired concentration to give a bed volume of 175 mm^3 . The column was placed in an incubator to maintain temperature, equilibrated with 350 ml aqueous gallotannin solution and used for bioconversion. Aqueous gallotannin solution (100 ml) was passed through column at a desired flow rate and the eluate was collected. Unless otherwise stated, the eluate was adjusted to pH 6 with ammonium hydroxide solution (10%) and was recycled. After each cycle, a sample of eluate was analyzed for residual gallotannin and gallic acid formed following the method described earlier¹⁷. Amount of tannase liberating $1 \mu\text{g}$ of gallic acid $\text{ml}^{-1} \text{ min}^{-1}$ was taken as unit activity. To study the storage stability of immobilized fungal mycelia, the column was unpacked, the beads were thoroughly washed with distilled water and stored in 0.02 M calcium chloride solution at 4°C till further use.

Effect of some important parameters on hydrolysis of gallotannin to gallic acid by calcium alginate entrapped fungal mycelia in a packed bed bioreactor has been presented in Table 1. Per cent hydrolysis of gallotannin was calculated on the basis of gallic acid content of the eluate after fifth cycle because only marginal increase in the gallic

Table 1—Effect of some parameters on the hydrolysis of gallotannin to gallic acid by calcium alginate entrapped fungal mycelia in a packed bed bioreactor

Parameters*	Per cent hydrolysis after 5 th cycle by entrapped mycelia of			
	<i>A. niger</i>	<i>A. fischerii</i>	<i>F. solani</i>	<i>T. viride</i>
Initial pH				
4	25.1	32.0	37.6	40.1
5	28.1	37.5	45.1	46.5
6	27.8	42.1	38.0	39.7
Temperature ($^\circ\text{C}$)				
25	10.1	22.1	25.5	36.6
35	18.3	43.9	50.4	46.6
45	14.4	42.6	44.4	51.1
Gallotannin conc. (g L^{-1})				
10	18.6	40.2	42.8	46.7
20	19.2	6.5	16.9	34.8
30	9.8	3.5	8.9	15.4
Residence time (min)				
175	15.1	30.0	44.1	42.2
87	18.2	26.9	41.7	50.4
60	10.7	5.4	25.9	50.8

*Except a parameter under investigation, the other operational conditions were undertaken at initial pH, 6; temperature, 35°C ; gallotannin concentration, 10 g^{-1} and residence time, 175 min

Table 2—Retention of tannase activity in the immobilized fungal mycelia stored at 4°C

Organism	Per cent reduction of tannase activity after (days)				
	30	60	90	120	150
<i>A. niger</i>	93.45	86.93	66.80	57.86	58.00
<i>A. fischerii</i>	90.34	84.11	59.35	41.85	26.80
<i>F. solani</i>	100.00	97.75	95.94	84.52	82.97
<i>T. viride</i>	92.50	86.39	86.79	86.00	85.07

Operational conditions are same as in Table 1

acid content was recorded thereafter for several cycles in all the cases.

It is evident from Table 1 that immobilized fungal mycelia exhibited maximum hydrolysis at initial pH 5, except *A. fischerii*, where the maximum conversion was obtained at pH 6. pH of the reaction medium showed continuous shift towards acidic as the hydrolysis progressed due to accumulation of gallic acid. Also the source of gallotannin is known to alter pH optima of tannase¹⁸. A relatively wider range of pH stability between 3.5 and 7.5 of tannase enzyme^{19,20} appears to be a desirable character under the condition of continuous change in pH of the reaction medium. Similar variation in hydrolytic activity of immobilized fungal mycelia was observed at different temperatures. *A. niger*, *A. fischerii* and *F. solani* produced maximum gallic acid at 35°C, whereas, *T. viride* gave higher conversion at 45°C.

Gallotannins are known to form complexes with proteins and inactivate the enzyme system of organism²¹. Higher concentration of gallotannin is, therefore, expected to inhibit its hydrolysis. Inhibition of tannase enzyme was clearly evident in case of *A. fischerii*, *F. solani* and *T. viride*. A slight increase in tannase activity of *A. niger* at 20 g L⁻¹ concentration indicated the tolerance of this fungus to higher concentration of gallotannin as compared to rest of the fungi. Increase in the residence time from 87 to 175 min increased hydrolysis of gallotannin in case of *A. fischerii* and *F. solani*, whereas, in *A. niger* maximum bioconversion was observed at a residence time of 87 min. *T. viride* exhibited peak bioconversion at 60 min residence time.

Operational and storage stability of immobilized cells are decisive factors for successful commercialization of the process. An experiment conducted for estimating the loss of tannase activity during continuous column operation for eight runs revealed no loss of tannase activity in *A.*

fischerii, 7.5% loss in *A. niger* and 18% loss in *F. solani* and *T. viride*. Swelling of alginate beads was observed after first two runs in a column due to removal of Ca²⁺ ions from calcium alginate by acidic gallotannin solution. Intermittent curing of gel beads in calcium chloride solution (0.02 M) prevented swelling and subsequent breakage of gel beads.

Retention of tannase activity of immobilized fungal mycelia stored at 4°C for different time periods has been shown in Table 2. All fungi selected for the present study showed less than 10% tannase activity loss after 30 days of storage. However, rate of loss of tannase activity was faster in *A. fischerii* and *A. niger* as compared to rest two fungi on prolonged storage. *F. solani* and *T. viride* mycelia entrapped in calcium alginate gel beads retained maximum tannase activity even after 150 days of storage and these fungi may be further explored for continuous hydrolysis of gallotannins to gallic acid in a packed bed bioreactor.

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