0273-2289/02/102–103/0071/$12.25

Continuous Production of Extracellular L-Glutaminase by Ca-Alginate-Immobilized Marine *Beauveria bassiana* BTMF S-10 in Packed-Bed Reactor

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Abstract

L-Glutamine amidohydrolase (L-glutaminase, EC 3.5.1.2) is a therapeutically and industrially important enzyme. Because it is a potent antileukemic agent and a flavor-enhancing agent used in the food industry, many researchers have focused their attention on L-glutaminase. In this article, we report the continuous production of extracellular L-glutaminase by the marine fungus *Beauveria bassiana* BTMF S-10 in a packed-bed reactor. Parameters influencing bead production and performance under batch mode were optimized in the order-support (Na-alginate) concentration, concentration of $CaCl_2$ for bead preparation, curing time of beads, spore inoculum concentration, activation time, initial pH of enzyme production medium, temperature of incubation, and retention time. Parameters optimized under batch mode for L-glutaminase production were incorporated into the continuous production studies. Beads with 12×10^8 spores/g of beads were activated in a solution of 1% glutamine in seawater for 15 h, and the activated beads were packed into a packed-bed reactor. Enzyme production medium (pH 9.0) was pumped through the bed, and the effluent was collected from the top of the column. The effect of flow rate of the medium, substrate concentration, aeration, and bed height on continuous production of L-glutaminase was studied. Production was monitored for 5 h in each case, and the volumetric productivity was calculated. Under the optimized conditions for continuous production, the reactor gave a volumetric productivity of 4.048 U/(mL·h), which indicates that continuous production of the enzyme by Ca-alginate-immobilized

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spores is well suited for *B. bassiana* and results in a higher yield of enzyme within a shorter time. The results indicate the scope of utilizing immobilized *B. bassiana* for continuous commercial production of L-glutaminase.

Index Entries: Glutaminase; *Beauveria bassiana*; immobilization; packed-bed reactor.

Introduction

L-Glutamine amidohydrolase (L-glutaminase, EC 3.5.1.2) is a therapeutically and industrially important enzyme *(1)*. Because it is a potent antileukemic agent *(2)* and a flavor-enhancing agent used in the food industry *(3)*, many researchers have focused their attention on L-glutaminase.

Marine microorganisms have the important characteristic of salt tolerance *(4)*, which renders use of them or their enzymes important in industrial processes that require high salt environments. Recently, we reported the production of salt-tolerant L-glutaminase by the marine fungus *Beauveria bassiana* BTMF S-10 under solid-state fermentation on polystyrene as an inert support *(5)*.

Although there are reports on the use of yeast cells for continuous conversion of glutamine to glutamate *(6)*, there are no previous reports on the production of L-glutaminase by an immobilized marine fungus. In this article, we report the continuous production of extracellular L-glutaminase by the marine fungus *B. bassiana* BTMF S-10 in a packed-bed reactor.

Materials and Methods

Microorganism and Preparation of Inoculum

B. bassiana isolated from the marine sediments of Cochin was used. Conidial inoculum was prepared from freshly raised 12-d-old cultures on Bennets agar media prepared in aged sea water (35 ppt) as described by Sabu et al. *(5)*.

Immobilization and Enzyme Production

Each 50 mL of the inoculum with the desired concentration of Conidia was mixed with 100 mL of a solution of Na-alginate of desired concentration. The mixture was extruded into $CaCl_2$ solution so that beads of an average diameter of 4 mm were formed. The beads were cured for 2 h in the same solution unless otherwise indicated and were preserved in saline until used. For enzyme production studies, the beads were activated in a solution of 1% glutamine in seawater prior to inoculation. Process parameters influencing the enzyme production by immobilized *B. bassiana* spores were optimized under batch mode in which 20 g of beads was inoculated in 50 mL of enzyme production medium (EPM) *(5)* in 250-mL Erlenmeyer flasks.

Optimization of Parameters Under Batch Process for Immobilization and Enzyme Production

Consecutive evaluation of parameters *(7)* was adopted for optimization of process parameters. Initially one parameter was evaluated, and it was incorporated at its optimized level in the subsequent experiment. Parameters influencing bead production and performance were optimized in the order-support (Na-alginate) concentration (1.5–5% [w/v]), concentration of $CaCl_2$ for bead preparation. (0.05–0.4 *M*), curing time of beads (1–5 h), spore inoculum concentration (2×10^8 to 16×10^8 spores/g of beads), activation time (5–25 h), initial pH of EPM (5.0–10.0 adjusted with 1 *N* HCl or NaOH), temperature of incubation (22–37°C), and retention time (6–42 h).

Continuous Production of L-Glutaminase by Ca-alginate Immobilized Beauveria Spores in Packed-Bed Reactor

Parameters optimized under batch mode for L-glutaminase production were incorporated into the continuous production studies. Beads with 12×10^8 spores/g of beads were activated in a solution of 1% glutamine in seawater for 15 h, and the activated beads were packed into a glass column (2.3-cm radius and 20-cm height) to a height of 15 cm. The top of the bed was covered with a perforated Teflon disc. EPM (pH 9.0) was pumped using a peristaltic pump from the bottom, and the effluent was collected from the top of the column. Air was introduced through a sparger at the bottom of the column after passing through a 22-µ bacterial filter (Millipore). Rate of aeration was monitored using a flowmeter (Eyela, Japan). Samples were collected at 1-h intervals and assayed for enzyme activity. The effect of flow rate of the medium (20–60 mL/h), substrate concentration (0.125–1.0%), aeration rate (0.4–1.7 vvm), and bed height (5–15 cm) on continuous production of L-glutaminase was studied. Production was monitored for 5 h in each case, and the volumetric productivity was calculated.

Enzyme Assay

L-Glutaminase activity was determined by the method of Imada et al. *(8)*. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µmol of NH_4 under optimal assay conditions. Enzyme yield was expressed as units per milliliter of EPM.

Results and Discussion

L-Glutaminase production by *B. bassiana* was observed under submerged culture conditions and under solid-state fermentation on an inert polystyrene support *(5)*. These observations led to investigation of the potential of Ca-alginate-immobilized *B. bassiana* for continuous production of the enzyme toward developing an ideal bioprocess for industrial pro-

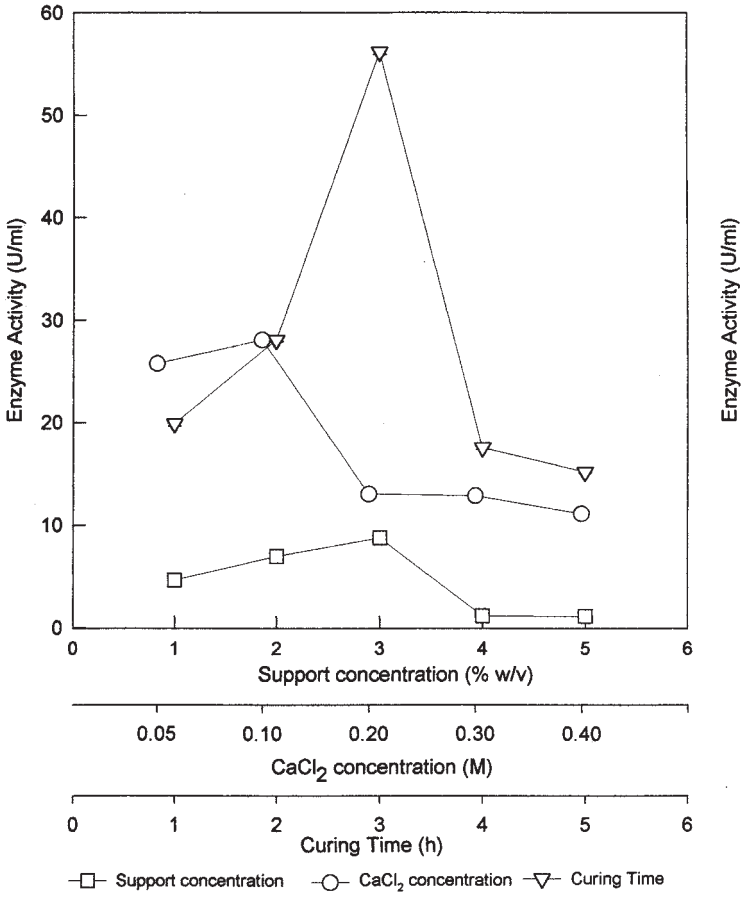

Fig. 1. Effect of support concentration, $CaCl_2$ concentration, and curing time on enzyme production by immobilized *B. bassiana*.

duction of the enzyme in which biomass can be recycled. Under the optimized conditions for continuous production, the reactor gave a volumetric productivity of 4.048 U/(mL·h), which indicates that continuous production of the enzyme by Ca-alginate-immobilized spores is well suited for *B. bassiana* and results in a higher yield of enzyme within a shorter time. This indicates the scope of utilizing immobilized *B. bassiana* for continuous commercial production of L-glutaminase.

Optimization of Process Parameters for L-Glutaminase Production by Ca-alginate-Immobilized Beauveria *sp. Spores in Batch Culture*

The data presented in Fig. 1 indicate the importance of the parameters support concentration, concentration of $CaCl_2$ solution used for bead production, and bead curing time on L-glutaminase production. At lower support concentrations, beads were malformed and unstable, with consid-

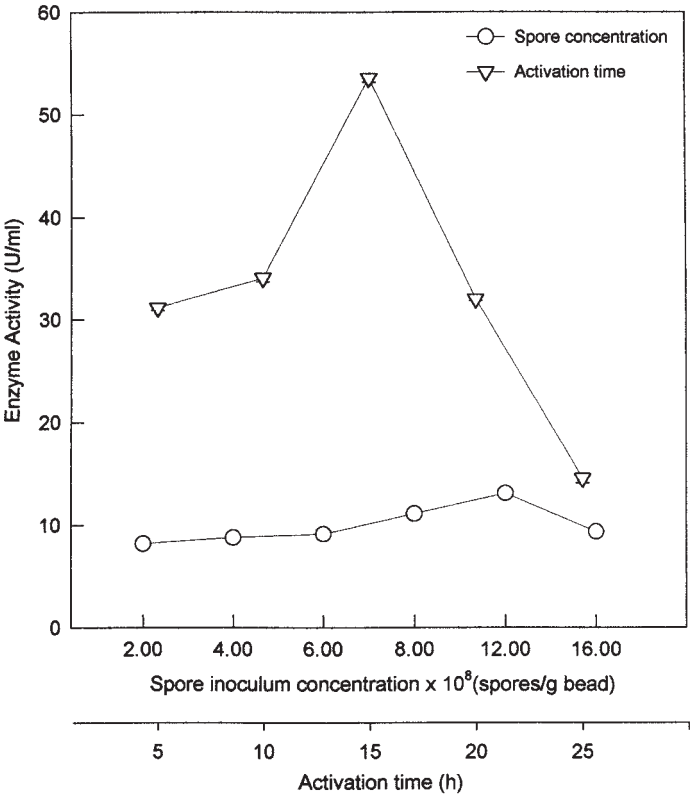

Fig. 2. Effect of spore inoculum concentration and bead activation time on enzyme production by immobilized *B. bassiana*.

erable leaching of spores. Enzyme yield increased with an increase in the support concentration up to 3% (w/v) (8.78 U/mL) and then showed a decline. This might be owing to the diffusional limitations encountered at higher alginate concentrations, since it is known that an increase in alginate concentration results in a tighter crosslinking *(9)*. The optimal concentration of $CaCl_2$ for the preparation of beads with maximal enzyme yield (28.08 U/mL) was 0.1 *M*. Nevertheless, considerable enzyme yield (25.74 U/mL) was also obtained when a 0.05 *M* solution was used for bead preparation. However, at that concentration, the beads formed were less stable with leaching of spores and floated in the solution. It was also noted that the beads required a curing time of 3 h to give maximum productivity (56.16 U/mL). An increase in curing time beyond 3 h drastically reduced the enzyme activity. Axelsson and Persson *(10)* have indicated that the process of gelation induces shrinking in Ca-alginate gel, which can cause diffusional limitations with increased crosslinking.

Inoculum concentration plays a significant role in immobilized whole-cell reactors. Of the different concentrations of spore inoculum tested, a concentration of 12×10^8 spores/g of beads gave the maximal enzyme yield of 13.1 U/mL (Fig. 2). The enzyme yield increased with an increase in spore

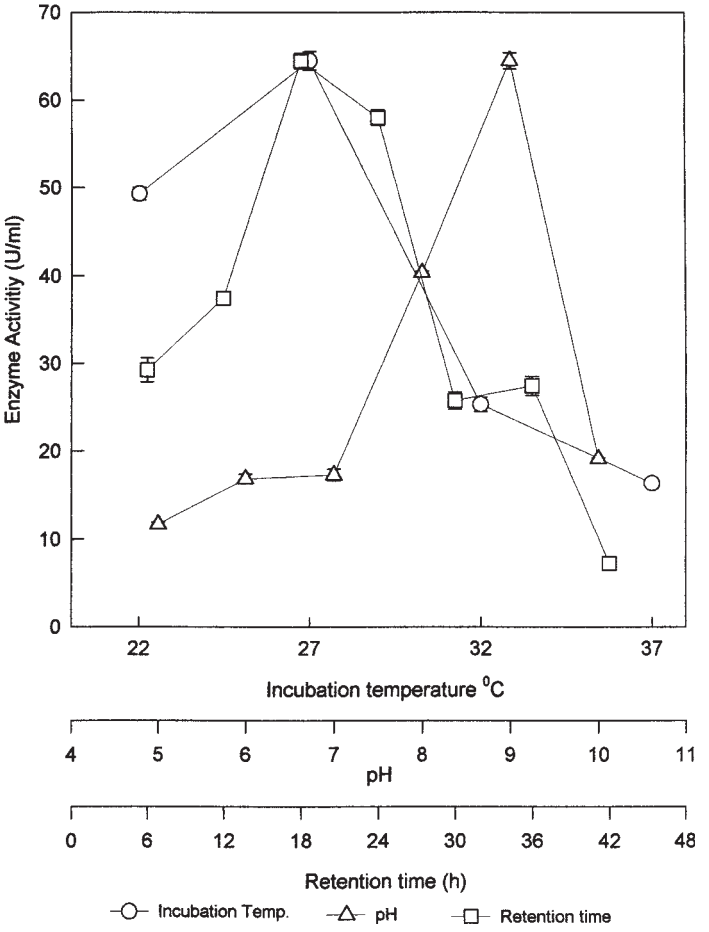

Fig. 3. Effect of incubation temperature, pH of medium, and retention time on enzyme production by immobilized *B. bassiana*.

inoculum concentration, characteristic of the immobilized-cell fermentations. However, there was a decrease in enzyme yield with the higher spore concentration of 16×10^8 spores/g of beads, which possibly is owing to substrate limitations at that spore concentration. Figure 2 also shows the effect of the duration of activation of the saline-preserved beads. Beads stored in saline at 4°C required an optimal activation of 15 h at 27 ± 2°C in 1% glutamine in seawater to bring them into the production phase. Fifteen hours of activation yielded an enzyme activity of 58.05 U/mL. A decrease in enzyme yield of beads activated for more than 15 h may have resulted because the beads had already entered the production phase while in activation medium and the enzyme activity could not be recovered from the EPM.

It was observed that the immobilized *B. bassiana* produced maximal enzyme at a pH of 9.0 and at room temperature (27 ± 0.2°C) (Fig. 3). The enzyme yields were 64.46 and 64.46 U/mL, respectively. The fungus also

Table 1
Reactor Performance at Different Flow Rates[a]

Flow rate (mL/h)	Dilution rate (h^{-1})	Residence time (h)	Enzyme yield (U/mL)	Productivity (U/[mL·h])
20	0.33	3.00	20.24	6.75
30	0.50	2.00	19.89	9.95
40	0.67	1.50	15.55	10.37
50	0.83	1.20	14.26	11.88
60	1.00	1.00	8.46	8.46

[a]Conditions: 15-cm bed height, 60-mL reactor void volume, 0.25% (w/v) substrate concentration.

recorded the maximal enzyme production at pH 9.0 under solid-state fermentation using polystyrene as inert support *(5)*. This may be owing to the alkalophilic nature of the fungus *(11)*. However, considerable enzyme production was recorded over a wide range of pH and temperature. These factors are largely characteristic of the organism and irrespective of the type of fermentation system employed *(12)*. The results in Fig. 3 also indicate that the optimal retention time for maximal enzyme yield (64.46 U/mL) by immobilized *B. bassiana* spores is 18 h. Beyond 18 h there was a probable inactivation of the enzyme, and the yield was reduced significantly.

Continuous Production of L-Glutaminase by Ca-alginate-Immobilized Spores of Beauveria sp. in Packed-Bed Reactor

Packed-bed reactors are the most widely studied bioreactor systems for immobilized cellular processes *(13)* and most ideal when relatively longer retention times are required and external biomass buildup is minimal. Hence, we conducted our studies on continuous production of L-glutaminase in a packed-bed reactor incorporating the optimal level of parameters obtained in batch production studies and evaluated the parameters that directly influenced reactor performance.

Effect of Flow Rate

Of the different flow rates tried (20–60 mL/h), the lowest flow rate of 20 mL/h gave the maximum productivity for glutaminase (4.048 U/[mL·h]) and showed a linear decline with increase in flow rate (Table 1). This may be attributed to the fact that at lower flow rates, the residence time is longer and therefore the beads are in contact with the medium for a longer duration.

Effect of Substrate Concentration

Often in continuous production, the medium is in contact with the immobilized cells for a relatively shorter duration, and unless recycled, most of the substrate remains unutilized. Thus, a study was performed to

Table 2
Reactor Performance at Different Substrate Concentrations[a]

Substrate conc. (% [w/v])	Dilution rate (h^{-1})	Residence time (h)	Enzyme yield (U/mL)	Productivity (U/[mL·h])
0.125	0.33	3.00	9.89	3.30
0.25	0.33	3.00	20.24	6.75
0.50	0.33	3.00	14.62	4.87
1.0	0.33	3.00	16.61	5.54

[a]Conditions: 15-cm bed height, 60-mL void volume, 20 mL/h flow rate.

Table 3
Effect of Aeration on Reactor Performance[a]

Aeration rate (vvm)	Dilution rate (h^{-1})	Residence time (h)	Enzyme yield (U/mL)	Productivity (U/[mL·h])
0	0.33	3.00	20.24	6.75
0.4	0.33	3.00	17.96	5.99
0.70	0.33	3.00	15.42	5.14
1.04	0.33	3.00	15.09	5.03
1.32	0.33	0.01	4.91	1.64
1.7	0.33	0.01	0	0.00

[a]Conditions: 15-cm bed height, 20 mL/h flow rate, 60-mL void volume, 0.25% (w/v) substrate concentration.

determine the optimal substrate concentration in the enzyme production medium at a flow rate of 20 mL/h. It was found that the maximum enzyme productivity (4.048 U/[mL·h]) was observed at a lower substrate concentration of 0.25% (w/v) (Table 2). This is a clear advantage since in submerged *(14)* or solid-state fermentation *(5)* a higher substrate concentration is required for the same organism to give comparable yields.

Effect of Aeration Rate

Because *Beauveria* is an aerobic organism, the effect of aeration rate on enzyme production was determined by aerating the packed-bed reactor at different levels, while being operated at a flow rate of 20 mL/h. Interestingly, it was found that the maximum enzyme production (4.048 U/[mL·h]) was obtained in the absence of forced aeration, and any increase in aeration rates resulted in a decrease in enzyme productivity (Table 3). This could be owing to the fact that air bubbles fill the voids of beads, thereby reducing the contact between the beads and the medium.

Effect of Reactor Bed Height

Increase in bed heights had a clear advantage in the productivity of the reactor with the highest bed height (15 cm) giving the maximum produc-

Table 4
Reactor Performance at Different Bed Heights[a]

Bed height (cm)	Void volume (mL)	Dilution rate (h^{-1})	Residence time (h)	Enzyme yield (U/mL)	Productivity (U/[mL·h])
5	10	2.00	0.50	15.442	30.88
10	20	1.00	1.00	27.84	27.84
15	60	0.33	3.00	20.242	6.75

[a]Conditions: 20 mL/h flow rate, 0.25% (w/v) substrate concentration.

tion (4.45 U/[mL·h]) and the lowest bed height (5 cm) giving the lowest production (3.088 U/[mL·h]) (Table 4) for the given flow rate. This indicates that the increase in residence time with the increase in bed height would in turn increase the contact time between the beads and the medium and thereby effect a higher productivity.

Conclusion

Under the optimized conditions for continuous production, the reactor gave a volumetric productivity of 4.048 U/(mL·h), which indicates that continuous production of the enzyme by Ca-alginate-immobilized spores is well suited for *B. bassiana* and results in a higher yield of enzyme within a shorter time. This indicates the scope of utilizing immobilized *B. bassiana* for continuous commercial production of L-glutaminase.

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