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# Continuous production of L-glutaminase by an immobilized marine *Pseudomonas* sp BTMS-51 in a packed bed reactor

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**Abstract**

A marine *Pseudomonas* sp BTMS-51, immobilized by Ca-alginate gel entrapment was used for the production of extracellular L-glutaminase under repeated batch process and continuous process employing a packed bed reactor (PBR). Immobilized cells could produce an average of 25 U/ml of enzyme over 20 cycles of repeated batch operation and did not show any decline in production upon reuse. The enzyme yield correlated well with the biomass content in the beads. Continuous production of the enzyme in PBR was studied at different substrate concentrations and dilution rates. In general, the volumetric productivity increased with increased dilution rate and substrate concentrations and the substrate conversion efficiency declined. The PBR operated under conditions giving maximal substrate conversion efficiency gave an average yield of 21.07 U/ml and an average productivity of 13.49 U/ml/h. The system could be operated for 120 h without any decline in productivity.


*Keywords:* Glutaminase; Immobilization; Packed bed reactor; Marine bacteria; *Pseudomonas*; Continuous fermentation

## 1. Introduction

Microbial glutaminases have found applications in several fields. They had been tried as therapeutic agents in the treatment of cancer [1,2] and HIV [3], as an analytical agent in determination of glutamine and glutamate [4–6], and in the production of specialty chemicals like threonine by gamma glutamyl transfer reactions [7]. However, one of the major uses of microbial glutaminase is in the food industry, where it is used as a flavour enhancing agent [8–10].

Salt and thermo tolerant glutaminases are particularly desired in the food industry, and naturally occurring marine microorganisms have become an ideal source to look for the salt tolerant enzyme. Indeed there have been several attempts for producing halo tolerant glutaminases from marine bacteria and fungi [11–13]. Marine bacteria are adapted to live attached to submerged surfaces, which often results in biofouling, but at the same time this feature is advantageous for immobilized cell processes. It was recognized from earlier studies that marine bacteria anchored to a nutritionally inert support—polystyrene was capable of secreting extracellular L-glutaminase when cultured in a seawater based medium [12]. The marine bacterium *Pseudomonas* sp BTMS-51, was found to produce more extracellular glutaminase when immobilized by entrapment in Ca-alginate beads. The process conditions for immobilization and enzyme production by the bacterium were optimized under batch mode [14]. In the present study, we describe the possible effects of operational conditions on enzyme production and explore the possibility of continuous production of the enzyme by immobilized cells of *Pseudomonas* sp BTMS-51 in a packed bed reactor (PBR).

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## 2. Materials and methods

### 2.1. Microorganism

*Pseudomonas* sp BTMS-51, available at the culture collection of Department of Biotechnology, Cochin University of Science and Technology, was used in the present study. The working culture was maintained on Zobell's Marine Agar slopes at 4 °C and was periodically sub-cultured.

### 2.2. Immobilization

A loop full of Zobell's agar slope culture of the bacterium was transferred to 10 ml of Zobell's Marine Broth and incubated overnight at 35 °C in a shaker incubator. The entire contents were transferred to 150 ml of the same medium in 500 ml Erlenmeyer flasks and incubated at 35 °C and 150 rpm agitation, for 18 h after which the entire contents were used to inoculate 1.5 l of Zobell's broth in a 3 l Lab Fermenter (Eyela, Japan). Incubation was further carried out with 300 rpm agitation and 150 cc/min aeration at 35 °C for 18 h, after which the medium was centrifuged at 6000 rpm for 10 min to recover the cells. The pellets were washed and suspended in sterile saline. The suspension was mixed with sterile Na-alginate solution so that the final slurry was 3% (w/v) with respect to alginate and 1% (w/v) with respect to the cell wet weight. The mixture was extruded through a fine micropipette tip attached to silicon tube, using a peristaltic pump into a sterile solution of 0.15 M $CaCl_2$ aseptically while being stirred, when spherical beads of an average diameter of 4 mm were formed. The beads were cured in the same solution for 2 h and stored in sterile saline containing 0.1% $CaCl_2$ at 4 °C until used.

### 2.3. Enzyme production medium

Seawater glutamine medium (SWG) optimized for L-glutaminase production by immobilized *Pseudomonas* sp cells under batch mode [14] with the composition L-glutamine-20 g, D-glucose-10 g, aged seawater-1 l, pH 6.0., was used as the enzyme production medium for repeated batch and continuous production of the enzyme. The substrate (L-glutamine) concentration was varied wherever indicated, in the continuous production experiments.

### 2.4. Repeated batch fermentation for L-glutaminase production

The suitability for reuse of the immobilized viable cell beads for L-glutaminase production was evaluated by using the cells repeatedly for 20 batches of operation. Twenty gram of the immobilized viable cell beads were activated by incubating in a 1% L-glutamine solution in aged seawater (pH 5.00) at room temperature (27 ± 2 °C) for 18 h. The activation medium was drained and the beads introduced into 50 ml of SWG in 250 ml Erlenmeyer flasks and incubated at 35 °C for 12 h in a shaker incubator at 80 rpm agitation. Medium after incubation was collected and used for determination of cell leaching (measured as total protein). A 10 ml sample was centrifuged at 6000 rpm, and 4 °C for 10 min to recover the supernatant, which was used for assaying enzyme activity. The beads were recovered aseptically, washed in sterile saline and were transferred to 50 ml of fresh SWG to continue the next cycle of operation. At the end of each cycle, five beads were withdrawn and biomass in the beads was estimated after dissolving in 0.5 M citrate buffer (pH 5.0) as total protein. The repeated batch operation was continued for 20 cycles and the performance of immobilized cells evaluated.

### 2.5. Continuous production of L-glutaminase in PBR

A glass column reactor of height 45 cm and internal diameter 3.6 cm was used for the experiment. The reactor had a bottom support of perforated glass. Activated beads were aseptically introduced into the reactor and packed to a height of 30 cm from the bottom disc. A perforated Teflon disc was placed over the beads to prevent bed expansion during operation. The void volume of the reactor was 94.4 cc. The reactor was operated at 30 °C in a temperature controlled room. Enzyme production medium was introduced from the top of the column and the effluent was collected from the bottom. The inlet and outlet flow rates were kept equal to maintain the liquid level constant at just above the bed level. The reactor was equilibrated by running at the desired feed rate and substrate concentration, for a minimum of 6–8 h before studying the enzyme production.

SWG medium containing different substrate concentrations (0.5, 1, 1.5 and 2% w/v) was used for the study. For each medium, the reactor was run at five different dilution rates (0.64, 0.85, 1.06, 1.27 and 1.48/h) and at each dilution rate the reactor was operated continuously for a minimum of 12 h after equilibration. Samples were withdrawn every 2 h and enzyme production and substrate concentration in the influent and effluent stream were analyzed. From these data, the volumetric productivity and substrate conversion efficiency of the reactor was calculated.

The suitability of the reactor for prolonged operation in the continuous mode was evaluated at the substrate concentration and feed rate which gave maximum substrate conversion efficiency over a period of 120 h (5 days) after equilibration.

### 2.6. Analytical methods

L-glutaminase activity was determined by the method of Imada et al. [15] with minor modifications [12]. One unit of glutaminase activity was defined as the amount of enzyme that liberates 1 μM of ammonia under standard assay conditions. Enzyme activity was expressed as U/ml of the sample. Cell leaching into the medium and biomass in the beads were estimated as total protein by the method of Herbert et al. [16]. For estimation of biomass in the Ca-alginate beads, they were dissolved in 1 M citrate buffer (pH 5.0) before assaying total protein. Biomass in the medium was estimated as mg protein/ml of medium and that in the beads as mg protein/g beads. From the values, the percentage of total biomass in beads and in medium was calculated and expressed as % biomass. Substrate (L-glutamine) concentration in the influent and effluent was measured using a glutamate dehydrogenase method [4] and substrate conversion efficiency was expressed as % conversion.

## 3. Results and discussion

### 3.1. Repeated batch fermentation

The data obtained for 20 cycles of repeated batch operation shown in Fig. 1 indicated that immobilized cells were active and could be reused, since the enzyme yield remained between 21 and 33 U over the entire period of operation. On an average 25 U/ml of L-glutaminase was synthesized per cycle. The biomass in the beads increased with the batches of operation for the first few cycles of operation and remained almost steady for the subsequent batches between 45 and 50% total protein (average 3.3-mg protein/g bead). Growth as free cells (leaching) did occur in the medium and accounted for 44–68% of the total biomass. Between 2.94 and 4.52 mg/ml of protein was recorded in the medium. The yield showed correlation with the biomass content in the beads, indicating that enzyme production was a function of cell concentration under the conditions of operation. It is recognized that the mode of operation has a profound influence on the immobilized cell processes, and during batch operation, the rapid changes in medium composition brought about by substrate depletion and accumulation of products, byproducts and toxic substances can have a negative impact on cell activity and multiplication [17]. This is overcome by incorporation of regeneration cycles of providing growth medium in between the production cycles. However, in the present study it was observed that substrate depletion or product accumulation did not exert a dominant influence on enzyme production within the short time of retention (12 h) employed in the cycles, and there was no reduction in the immobilized cell biomass after 20 cycles. The results showed potential for repeated reuse of immobilized cells without reduction in biomass and enzyme synthesis.

### 3.2. Continuous production of L-glutaminase in PBR

#### 3.2.1. Effect of dilution rate and substrate concentration on enzyme yield

Enzyme production by immobilized cells decreased with an increase in dilution rate, regardless of the substrate concentrations employed (Table 1). While

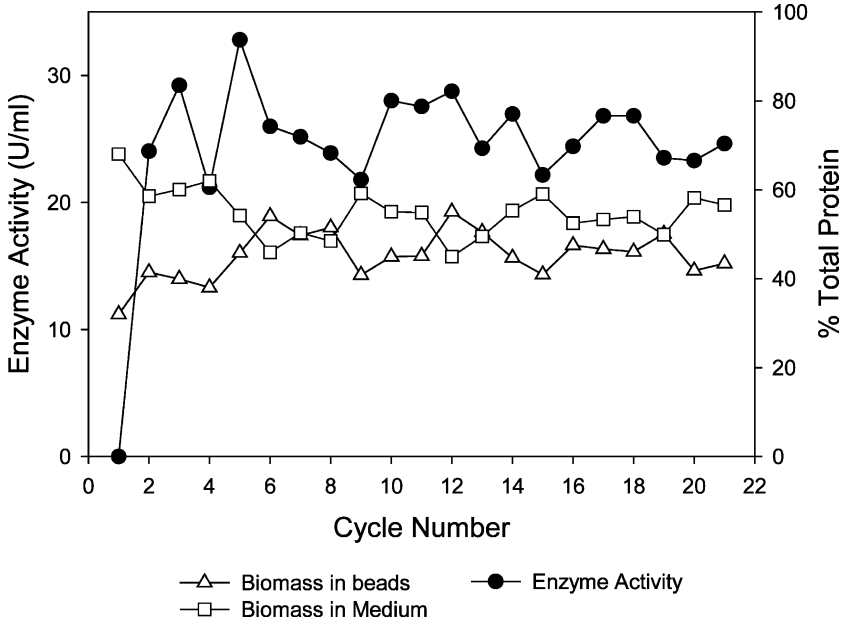


Fig. 1. Repeated batch production of L-glutaminase by Ca-alginate immobilized cells.

Table 1
Continuous production of glutaminase in PBR by immobilized cells

| Substrate concentration (% w/v) | Flow rate, $F$ (ml/h) | Dilution rate, $D = F/V$ (1/h) | Enzyme yield, $Y$ (U/ml) | % substrate conversion | Volumetric productivity, $P = YD$ (U/ml/h) |
|---|---|---|---|---|---|
| 0.5 | 60 | 0.64 | 19.68 | 28.10 | 12.51 |
| | 80 | 0.85 | 15.43 | 23.50 | 13.08 |
| | 100 | 1.06 | 13.52 | 22.77 | 14.32 |
| | 120 | 1.27 | 10.97 | 22.53 | 13.94 |
| | 140 | 1.48 | 8.29 | 17.93 | 12.29 |
| 1.00 | 60 | 0.64 | 27.56 | 18.05 | 17.52 |
| | 80 | 0.85 | 24.42 | 15.63 | 20.69 |
| | 100 | 1.06 | 20.49 | 14.39 | 21.71 |
| | 120 | 1.27 | 18.24 | 13.69 | 23.19 |
| | 140 | 1.48 | 15.32 | 13.20 | 22.72 |
| 1.50 | 60 | 0.64 | 35.52 | 11.14 | 22.58 |
| | 80 | 0.85 | 30.22 | 10.09 | 25.61 |
| | 100 | 1.06 | 25.21 | 9.37 | 26.71 |
| | 120 | 1.27 | 22.86 | 9.13 | 29.06 |
| | 140 | 1.48 | 18.44 | 8.80 | 27.35 |
| 2.00 | 60 | 0.64 | 36.05 | 8.84 | 22.91 |
| | 80 | 0.85 | 32.25 | 8.18 | 27.33 |
| | 100 | 1.06 | 27.27 | 7.69 | 28.89 |
| | 120 | 1.27 | 24.04 | 7.09 | 30.56 |
| | 140 | 1.48 | 20.52 | 6.12 | 30.43 |

Effects of substrate concentration and dilution rate on enzyme yield, volumetric productivity and substrate conversion efficiency.

the maximal enzyme yield (36.05 U/ml) was observed at a dilution rate of 0.64/h in the medium containing 2% w/v glutamine, the lowest yield (8.29 U/ml) was obtained at a dilution rate of 1.48/h in the medium containing the lowest substrate concentration (0.5% w/v). Enzyme yield showed a linear increase with increase in substrate concentration at all the dilution rates tested. The rate of synthesis of the enzyme by a given equilibrium biomass at a given dilution, may require a critical substrate concentration besides its requirement for cell growth and maintenance. With increase in dilution rate, the rate of substrate supplementation increases, product dilution is also a simultaneous occurrence, which is probably the reason for an observed decrease in activity with higher dilution rates. Further, the probable existence of diffusional effects in calcium alginate gels at higher dilution rates could also have contributed to the observed decrease in activity.

### 3.2.2. Effect of dilution rate and substrate concentration on volumetric productivity

Volumetric productivity of the reactor increased along with increase in dilution rates up to a rate of 1.27/h for the substrate concentrations 1, 1.5 and 2% whereas, at a substrate concentration 0.5%, this increase in productivity with dilution rate was observed only up to a dilution rate of 1.06/h (Table 1). Maximal productivity was recorded at a dilution rate of 1.27/h with a 2% w/v substrate concentration; and the lowest was recorded at a dilution rate of 0.64/h in medium containing 0.5% w/v substrate. At any given dilution rate, the increase in productivity was directly proportional to the substrate concentration. The availability of substrate may be considered as a key factor in enzyme production by the immobilized cells. With a substrate concentration of 0.5%, the cells probably were not able to attain their maximal rate of synthesis due to substrate limitation, and the increase in dilution rates beyond 1.06/h resulted in a lower productivity. The dilution rate at which maximal productivity is obtained for a given biomass can be increased by increasing the substrate concentration until the rate of synthesis becomes a limiting factor, which can bring up the volumetric productivity of the system. However, this may not always be desirable since the product stream is more diluted and substrate conversion may not be efficient at higher dilutions although the productivity increases.

### 3.2.3. Effect of dilution rate and substrate concentration on percentage substrate conversion

Maximal substrate conversion was obtained at the lower dilution rates employed, for all the individual substrate concentrations tested. The decrease in efficiency for substrate conversion was linear with dilution rates, irrespective of the concentration of substrate used in the medium (Table 1). The highest conversion rate that could be achieved was 28%, at a dilution rate of 0.64/h in a medium containing 0.5% substrate. Increase in substrate concentration resulted in decline of conversion rate regardless of the dilution rates tried and the

lowest conversion rates (6–12%) was recorded at 2% substrate concentration.

It may be noted that although the enzyme yield and productivity showed an increase with increase in substrate concentration, the system has a threshold level of utilization of the substrate for enzyme synthesis, which probably depends on the biomass content of the beads. The increase in yield probably results from the greater availability of substrate per unit surface area of the microbial cells for inducing the synthetic machinery of the cells. The choice whether to operate the reactor at maximal yield, output or substrate conversion efficiency has to be made judiciously, often depending upon process economy. There are reports where it was preferred to operate at a concentration that gave maximum substrate utilization, despite the fact that a higher productivity may not be achieved under such conditions [18].

### 3.3. Operational stability for the reactor

Studies on prolonged operation of the reactor were conducted at the substrate concentration and dilution rate, which gave maximal substrate conversion. The PBR operated at 0.5% w/v substrate concentration and a dilution rate of 0.64/h gave a consistent yield of L-glutaminase over 120 h of continuous operation. There was no apparent reduction in enzyme production and an average yield of 21.07 U/ml was recorded (Fig. 2). The biomass content of the beads also remained fairly constant, at an average 46% total protein (3.39 mg protein/g beads). However, there was leaching of cells and free cell growth in the medium. The % biomass content in the effluent stream remained between 41 and 62 (2.91 and 4.67 mg/ml protein) and average volumetric productivity was 13.49 U/ml/h. Operation under a low substrate concentration did not affect process stability at least for the tested duration, evident from the consistent average yield of enzyme. Further, it may also be noted that there was no significant reduction in the biomass content of the beads. Although in some cases the prolonged operation under continuous mode results in a sharp decline in productivity due to a reduction in biomass, it may be moderated by incorporating regenerative treatments with growth media between the production cycles [17]. In the present study, the system neither showed any sign of decay after 120 h of operation, nor warranted a regeneration treatment at least for the tested duration. This probably implies an attainment of equilibrium between growth of the cells within the beads and their death and detachment from it. Such a steady state is particularly desirable in continuous processes employing immobilized cells, eliminating the need for incorporation of a regeneration phase. Under such conditions the only limitation for continuous operation could be the stability of the beads.

### 3.4. Conclusions

Marine microorganisms differ very much from their terrestrial counterparts and are known to produce diverse spectra of novel useful metabolites [19]. Although it is very well recognized that marine bacteria are capable of adhesion to surfaces resulting in biofouling, this innate nature of adaptability to immobilized cell applications has rarely been utilized for metabolite production. The results presented in this paper indicate the potential for use of immobilized marine bacteria in

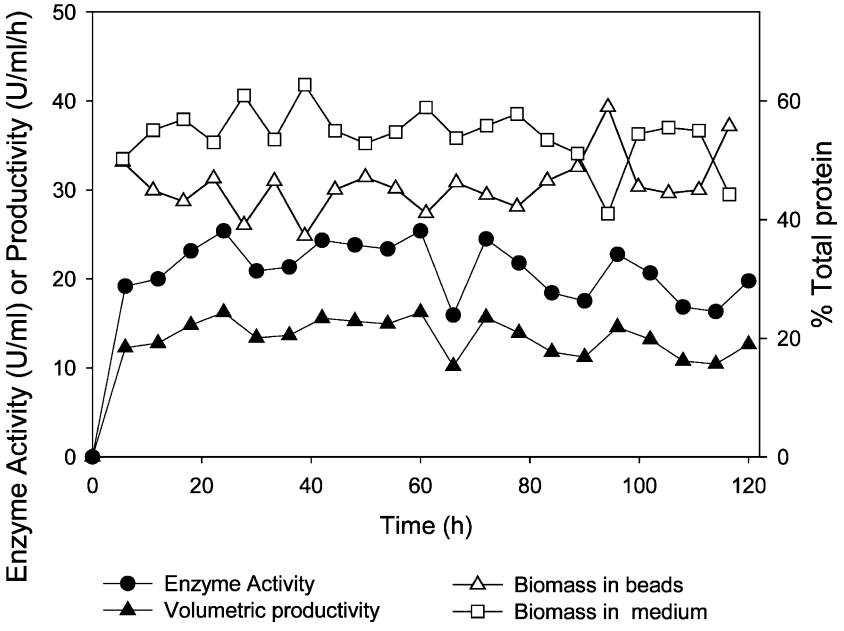


Fig. 2. PBR performance and variation of biomass concentration upon continuous production of L-glutaminase by Ca-alginate immobilized cells.

continuous production of extracellular enzymes. We believe that the present study is one of the first of its kind exploring the prospects of exploiting marine microbes employing immobilized cell technologies for production of economically useful microbial enzymes and metabolites.

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