

L-Glutaminase production by marine *Beauveria* sp. under solid state fermentation

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Abstract

Extracellular L-glutaminase production by *Beauveria* sp., isolated from marine sediment, was observed during solid state fermentation using polystyrene as an inert support. Maximal enzyme production (49.89 U/ml) occurred at pH 9.0, 27°C, in a seawater based medium supplemented with L-glutamine (0.25% w/v) as substrate and D-glucose (0.5% w/v) as additional carbon source, after 96 h of incubation. Enzyme production was growth associated. Results indicate scope for production of salt tolerant L-glutaminase using this marine fungus. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

L-Glutaminase (L-Glutamine amidohydrolase EC 3.5.1.2) has received significant attention owing to its potential as an anticancer agent [1] and a flavour enhancing agent in the food industry, as it increases the glutamic acid content of the food thereby imparting flavour [2]. Salt tolerant and heat stable L-glutaminase is in demand in the food industry [3,4]. Its commercial importance demands not only the search for better yielding viable strains, but also economically viable bioprocesses for its large-scale production [5]. In this context marine microorganisms hold significance by virtue of their ability to produce salt tolerant enzymes [6].

The production of extracellular L-glutaminase by marine *Pseudomonas* sp., *Vibrio costicola* [7–9], and *Micrococcus* sp. [3] was reported, whereas, in the case of fungi, except for reports on terrestrial *Aspergillus oryzae* [10,11] no information is available in the literature on extracellular L-glutaminase production by any marine fungi. Since the present source for this enzyme is limited to *A. oryzae* alone, a search for potential strains that hyper produce this enzyme with novel

properties and an economically viable bioprocess is pursued.

The use of nutritionally inert materials for solid state fermentation (SSF) facilitates accurate designing of media, monitoring of process parameters, scaling up strategies and various engineering aspects which are either impossible or difficult with conventional SSF using organic solid substrates such as wheat bran [12]. The inert materials, on impregnation with a suitable medium, provide a homogenous aerobic condition throughout the fermentor, and will not contribute impurities to the fermentation product [13]. This facilitates maximal recovery of the leachate with low viscosity and high specificity for the target product [8]. We reported earlier that polystyrene, a commercially available insulating and packaging material could be used as an inert solid support for the production of L-glutaminase by marine *V. costicola* under SSF [8,9]. While ion exchange resins [14], polyurethane foam [12,15] and computer cards [16] have been used as inert carriers for SSF with fungi, the use of polystyrene for SSF with fungi has not been reported.

In the present study, we evaluated the potential of *Beauveria* sp., an alkalophilic and salt tolerant fungus, isolated from marine sediment [17], for L-glutaminase production using polystyrene as solid support under solid state fermentation. To the best of our knowledge

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this is the first report on extracellular enzyme production using inert support under solid state fermentation by any marine fungi.

2. Materials and methods

2.1. Micro organism

The fungus *Beauveria* sp. BTMFS 10, isolated from marine sediment of Cochin [17] was used in the present study.

Conidial inocula were prepared from a freshly raised 12-day-old Bennet's agar (Himedia, India, prepared in aged seawater (35 ppt)) slope culture. The conidia were collected and suspended in 15 ml of 0.1% solution of Tween 80 (12×10^6 conidia/ml)

2.2. Pre-treatment of polystyrene beads

Commercially available expanded beads of polystyrene (diameter 4–5 mm) were initially subjected to pre-treatment [8], by autoclaving at 121°C for 15 min, when they collapsed to about one third of their original size. The beads were washed repeatedly in distilled water and dried overnight at 110°C. The beads thus prepared were used as inert support for solid state fermentation studies.

2.3. Enzyme production

Enzyme production was carried out in 500 ml Erlenmeyer flasks. Production medium containing g/l: L-glutamine-10, D-glucose-10, dissolved in sea water (salinity, 35 ppt), and pH adjusted to 8.0, was added to 10 g of polystyrene beads in the flasks at appropriate quantity so that the final moisture level after addition of inoculum was 80% v/w, at the time of commencement of incubation, unless otherwise specified. The flasks were autoclaved at 121°C for 15 min and were inoculated with the prepared inoculum at 10% (v/w) level, and incubated at 27°C, in an incubator, for 5 days with 90% relative humidity [17].

After incubation, the enzyme was extracted from the solid media in 0.1 M phosphate buffer (pH 8.0) by a simple contact method and the same was separated from the inert support by filtering through a cheese cloth. The separated solution was then centrifuged at 10 000 rpm, 4°C, 20 min for removing the mycelia [8]. The supernatant was used for enzyme assay.

2.4. Optimisation of process parameters for L-glutaminase production

The medium described above was taken as a basal medium and the process parameters under study were

varied. After optimisation of each parameter, it was included in the next study at its optimal level. The initial moisture content of the medium, initial pH of the medium (5–10), incubation temperature (22–47°C), additional NaCl concentration, additional nitrogen sources (peptone, yeast extract, beef extract, malt extract, ammonium sulphate, ammonium nitrate, calcium nitrate, and potassium nitrate at 1% w/v.), additional carbon sources (glucose, maltose, mannitol, lactose, sucrose and sorbitol at 1% w/v), and different aminoacids (L-glutamine, L-glutamic acid, L-asparagine, arginine, methionine, proline and lysine at 1% w/v) were optimised for L-glutaminase yield. After optimisation of process parameters the time course of L-glutaminase production was evaluated under the optimised conditions for a total period of 168 h. Growth of the fungus and enzyme production was monitored simultaneously in the time course experiment. After incubation for different time intervals, flasks were drawn and mycelial extraction was done by simple contact method using 0.1 M PO_4 buffer (pH 8) for 2 h on a rotary shaker at 300 rpm. The extract was used for measurement of enzyme activity. All experiments were conducted in triplicate and the mean values alone are reported.

2.5. Biomass estimation

Biomass was estimated in terms of total cell protein of the mycelia [8]. The mycelial pellet obtained, after centrifugation of the extract prepared from the polystyrene system was washed twice with sterile distilled water, resuspended in 10 ml of fresh sterile distilled water, mixed with 10 ml 3 N NaOH, heated in a boiling water bath for 5 min and cooled to room temperature. It was centrifuged at 10 000 rpm for 10 min and the supernatant was assayed for protein by a modified Lowry's method [18] and expressed as total cell protein (mg/ml).

2.6. Enzyme assays

Glutaminase was assayed according to Imada et al. [19]. The reaction mixture, containing 0.5 ml of L-glutamine (0.04 M), 0.5 ml of phosphate buffer 0.1 M (pH 8.0), 0.5 ml of distilled water and 0.2 ml of enzyme solution was incubated at 37°C for 30 min. The reaction was stopped by addition of 0.5 ml of 1.5 M Trichloroacetic acid. Then to 3.7 ml of distilled water, 0.1 ml of the above mixture and 0.2 ml of Nessler's reagent were added and colour developed was read after 10 min at 450 nm in a spectrophotometer (Spectronic-Genesys5). Enzyme and substrate blanks were used as controls. One unit of L-glutaminase activity was defined as the amount of enzyme that liberated 1 μmol of ammonia under optimal assay conditions. Assays

were done in triplicate and the mean enzyme activity was expressed as Units/ml of extraction buffer (U/ml).

3. Results and discussion

L-glutaminase production by a marine fungus *Beauveria* sp. under submerged culture condition was observed during the course of a previous study and the observation led to an investigation of the potential of L-glutaminase synthesis, as an exoenzyme, under solid

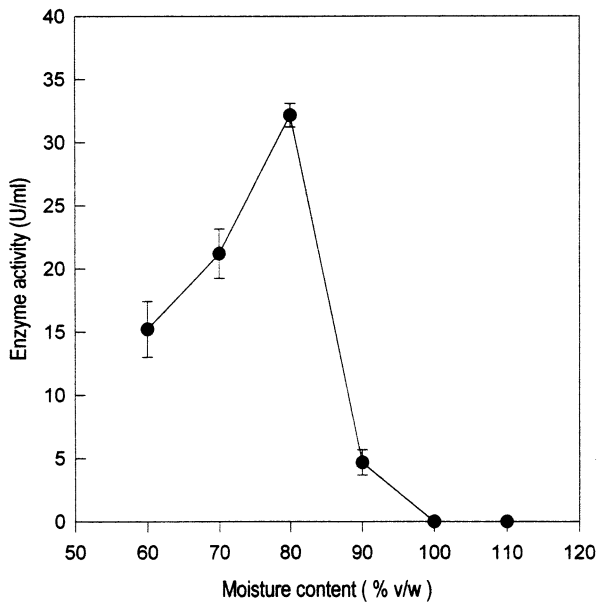


Fig. 1. Effect of initial moisture content on L-glutaminase production by *Beauveria* sp. under SSF.

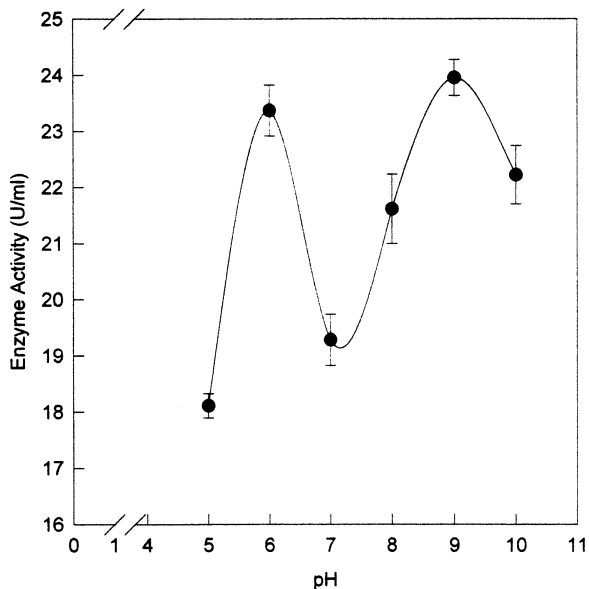


Fig. 2. Effect of initial medium pH on L-glutaminase production by *Beauveria* sp. under SSF.

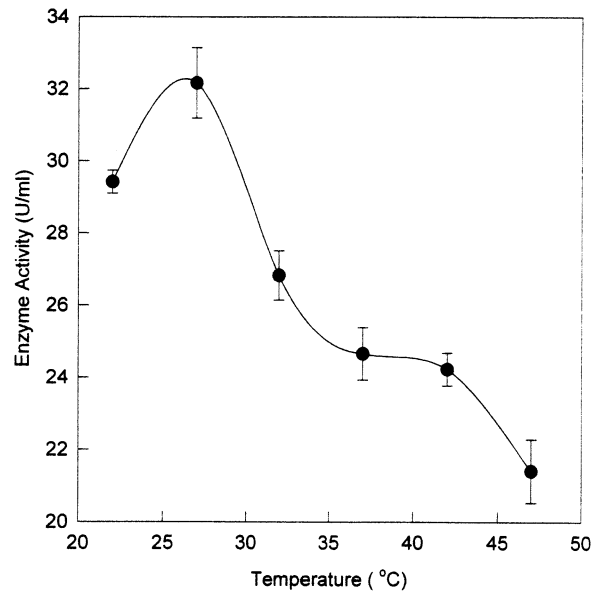


Fig. 3. Effect of incubation temperature on L-glutaminase production by *Beauveria* sp. under SSF.

state fermentation using polystyrene as an inert support, towards developing an ideal bioprocess for industrial production of this enzyme. Hence initially the various process parameters, which influence L-glutaminase production by *Beauveria* sp. under solid state fermentation conditions, were optimised. Data presented in Fig. 1 clearly indicate the influence of initial moisture content on L-glutaminase production by *Beauveria* sp. Enzyme production increased along with an increase in moisture content from 15.21 U/ml at 60% (v/w) to a maximum of 32.17 U/ml at 80% (v/w). Any further increase in the initial moisture content resulted in the existence of free water and consequent reduction in enzyme production. This observation is in agreement with that observed for the marine *V. costicola* [5]. The initial pH of the medium, at 27°C, influenced L-glutaminase production such that two pH optima were observed that favour high enzyme yield, one at pH 6.0 (23.37 U/ml) and another at pH 9.0 (23.96 U/ml) (Fig. 2). Most microbial extracellular enzymes are produced, at high levels, at a growth pH that is near to the optimal pH required for the maximal enzyme activity [20]. Incubation at 27°C, at pH 9.0 (optimised), enhanced enzyme production (32.17 U/ml) compared to other temperatures (Fig. 3). Nevertheless, a considerable level of enzyme production could be obtained at other pH and temperatures. These factors are largely characteristic of the organism and vary for each species [21].

Addition of NaCl to seawater in the enzyme production medium led to a decline in the enzyme production (Fig. 4). This indicates that the fungus is not halophilic, but could be halotolerant and a natural commensal

organism of the marine environment. A high NaCl concentration in the enzyme production medium through the addition of NaCl over and above that already present in the seawater, affected enzyme production. The data indicate that sea water could be an ideal medium or at least adequate to support L-glutaminase synthesis.

Incorporation of additional carbon sources enhanced enzyme yield from 25.52 to 32.02 U/ml (Fig. 5). Among the various carbon sources tested D-glucose (1%) pro-

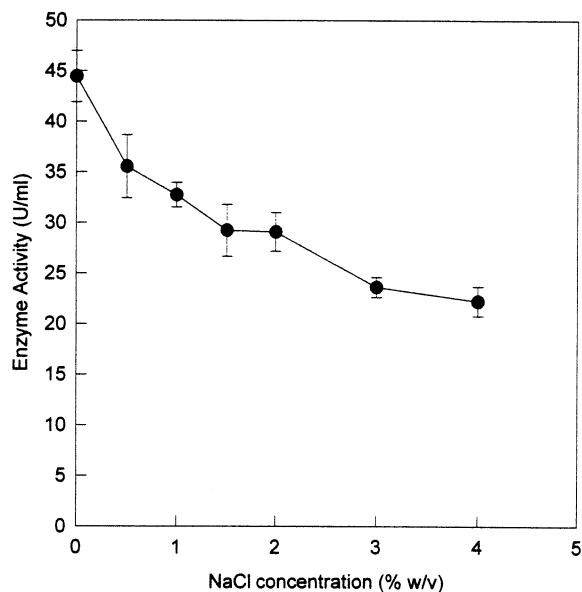


Fig. 4. Effect of additional NaCl concentration on L-glutaminase production by *Beauveria* sp. under SSF.

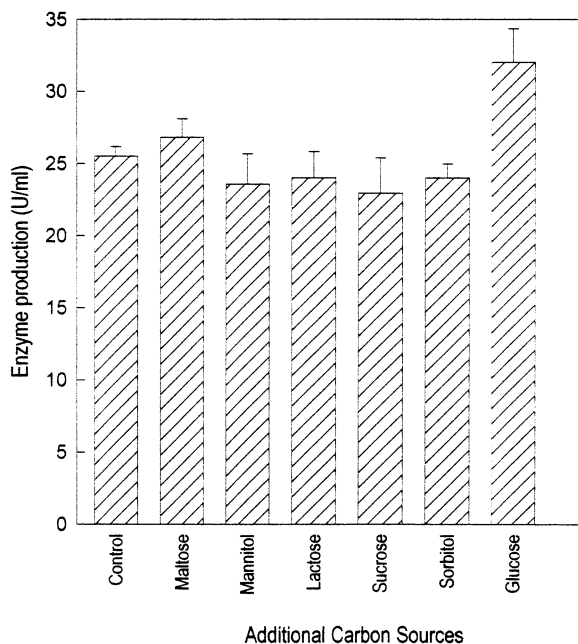


Fig. 5. Effect of additional carbon sources on L-glutaminase production by *Beauveria* sp. under SSF.

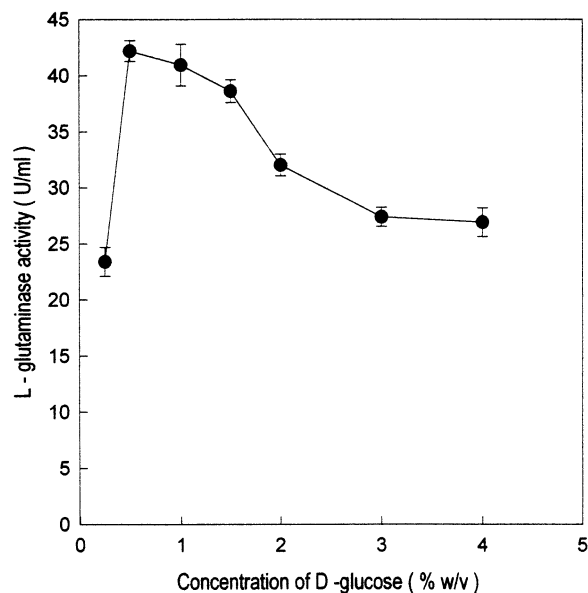


Fig. 6. Effect of D-glucose concentration on L-glutaminase production by *Beauveria* sp. under SSF.

moted maximal yield (32.02 U/ml) compared to others. Similar results were reported for the production of L-glutaminase by the marine *V. costicola* under solid state fermentation [5]. The enhanced production of L-glutaminase by incorporation of carbon sources may be attributed to the positive influence of additional carbon sources along with glutamine on enhanced biosynthesis. Optimal glucose concentration was studied by varying the glucose concentration in the medium and maximum enzyme production (42.12 U/ml) was observed at a concentration of 0.5% (w/v) (Fig. 6).

Nitrogen can be an important limiting factor in the microbial production of enzymes [21]. Among the different organic sources tested, Malt extract (1% w/v) was the best source for maximal enzyme production (31.59 U/ml) (Fig. 7). Further, the organic nitrogen sources, compared to inorganic nitrogen sources, at the given concentrations of nitrogen, promoted higher enzyme yield (31.59 U/ml), where a maximum production of 23.4 U/ml was observed with Potassium Nitrate (Fig. 8). Probably the presence of additional nitrogen sources along with L-glutamine in the medium promoted enhanced growth and consequent enzyme production.

Among the different amino acids tested, L-glutamine was observed to enhance L-glutaminase synthesis (32.17 U/ml) (Fig. 9). L-Glutaminase production (32.76 U/ml) occurred even in the absence of L-glutamine as well as any additional amino acid in the seawater medium (data not shown). This particular observation suggests that *Beauveria* sp. could produce extracellular L-glutaminase even in the absence of an enzyme inducer, when seawater was used as a medium. A detailed study on the molecular mechanism involved in the role of

seawater components in the biosynthesis of L-glutaminase would produce information on the biology of these organisms in natural environment alongside designing an economically viable fermentation media. The effect of glutamine concentration on production was evaluated in detail, as other amino acids and other nitrogen sources did not show enzyme yield higher than

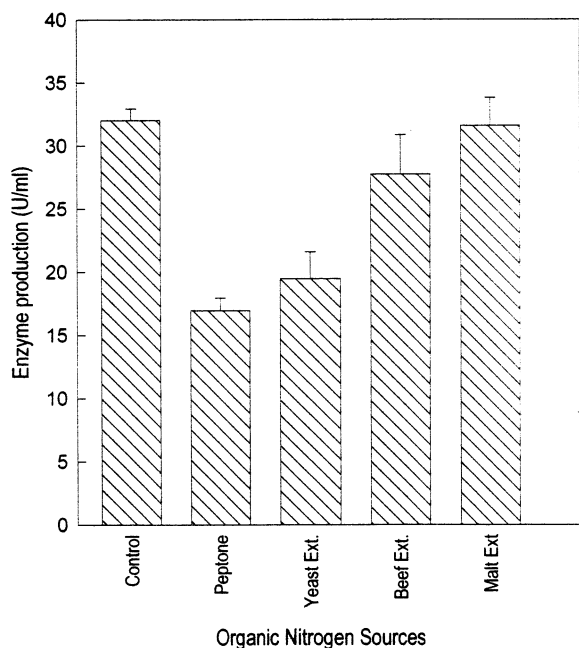


Fig. 7. Effect of organic nitrogen sources on L-glutaminase production by *Beauveria* sp. under SSF.

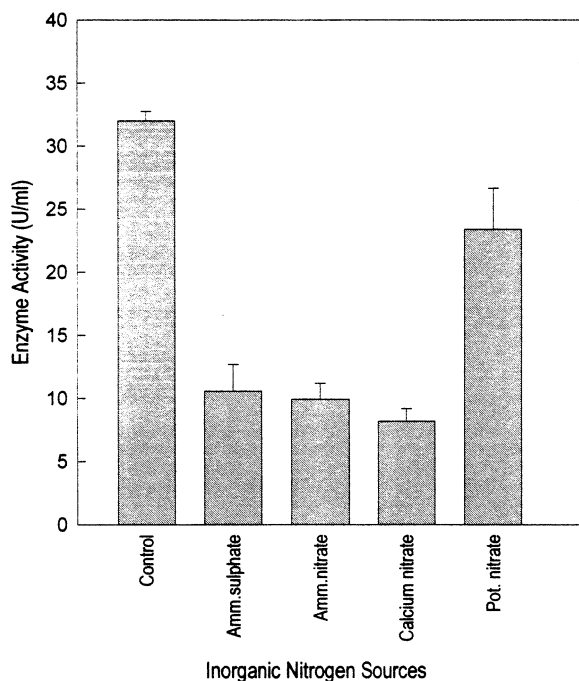


Fig. 8. Effect of inorganic nitrogen sources on L-glutaminase production by *Beauveria* sp. under SSF.

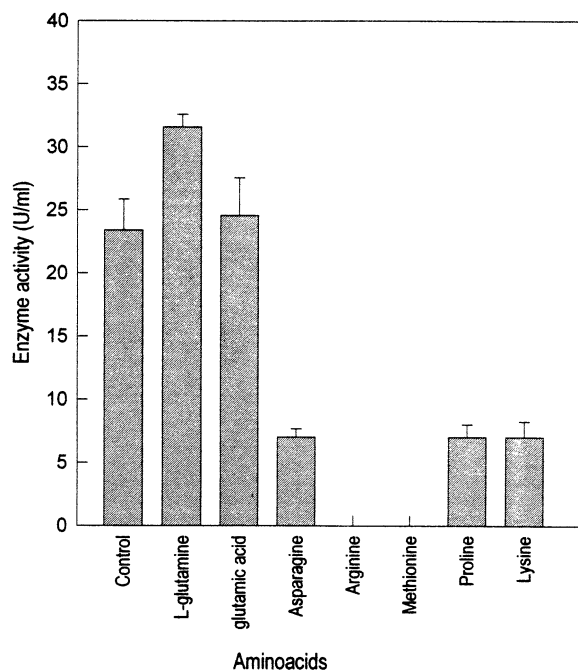


Fig. 9. Effect of different aminoacids on L-glutaminase production by *Beauveria* sp. under SSF.

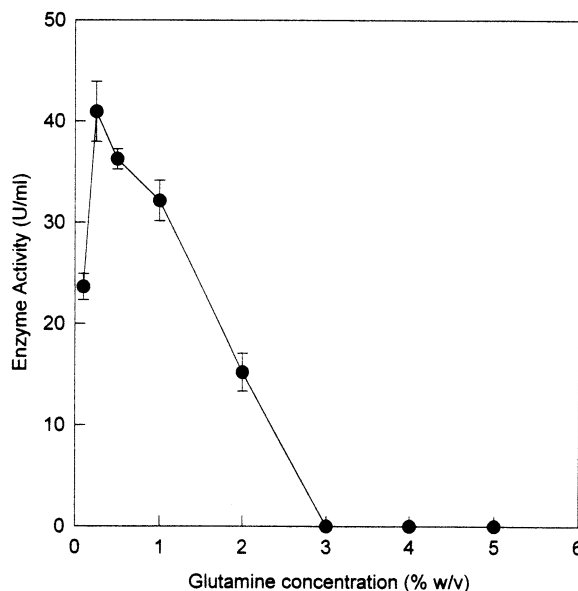


Fig. 10. Effect of L-glutamine concentration on L-glutaminase production. By *Beauveria* sp. under SSF.

that observed for glutamine. A maximal level of 40.95 U/ml was recorded at a concentration of 0.25% glutamine (Fig. 10)

After process optimisation, a time course study was conducted. Maximal enzyme production (49.89 U/ml) was observed with 80% (v/w) moisture content, at pH 9.0, 27°C, glucose (0.5%) as additional carbon source, and glutamine (0.25% w/v) as substrate, after 96 h of incubation. Enzyme production increased along with an

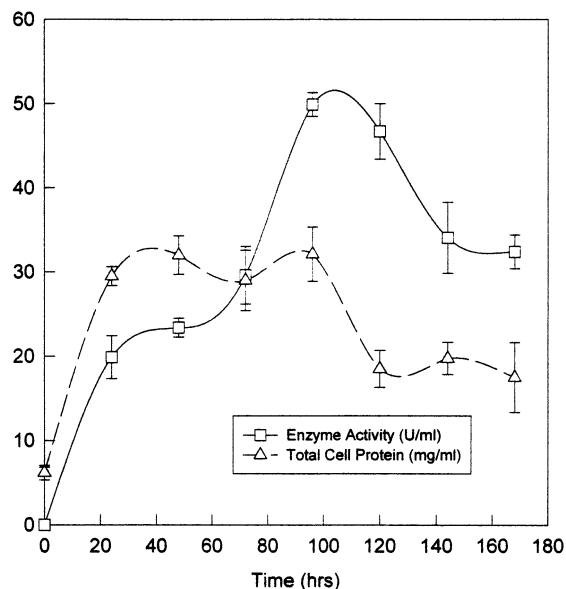


Fig. 11. Time course of L-glutaminase production by *Beauveria* sp. under SSF.

increase in total cell protein, during the logarithmic phase, up to 96 h followed by a decline on extended incubation (Fig. 11). This trend indicated that enzyme production was growth associated.

In conclusion, the results of the present study indicate scope for exploring marine fungi as a source for L-glutaminase, an enzyme that has gained industrial and pharmaceutical significance recently. Secondly, marine fungi can produce extracellular enzymes under SSF using polystyrene as an inert support. Thirdly seawater could provide the base for fermentation media for L-glutaminase production by marine fungi, under SSF.

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