Materials and Methods
2. MATERIALS AND METHODS

2.1. Microorganism

*Beauveria bassiana* BTMF S10 was used throughout the course of study.

2.1.1. Source of strain

The selected fungal strain was isolated from marine sediment of coastal environments of Cochin (Suresh, 1996) and is available as a stock culture in the culture collection of the Department of Biotechnology, Cochin University of Science and Technology, Cochin, India.

2.1.2 Maintenance of culture

The culture was maintained on Bennet's agar (HIMEDIA) slants and sub cultured once in a month. One set was maintained as stock culture preserved under sterile mineral oil. Another set was used as the working culture for routine experiments.

2.2 L-glutaminase production by marine *Beauveria bassiana* under Submerged fermentation (SmF).

2.2.1 Medium

Mineral salt glutamine medium with the composition given below was used as a basal medium (unless otherwise mentioned) for L-glutaminase production by *B. bassiana* under submerged fermentation.
The composition of the mineral salt glutamine medium (Renu and Chandrasekaran, 1992a) is as follows.

<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$</td>
<td>10</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>5</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>10</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>10</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>10</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>8.0</td>
</tr>
</tbody>
</table>

The prepared medium was autoclaved at 121°C for 15 minutes and used.

2.2.2 Preparation of inoculum

2.2.2.1 Spore inoculum

1. *Beauveria bassiana* was raised as agar slope culture on Bennet's agar, prepared in aged sea water, (in 50 ml capacity test tubes).

2. To fully sporulated (two weeks old) agar slope culture, 20 ml of sterile physiological saline (0.85 % NaCl) containing 0.1 % Tween 80 was added by means of a sterile pipette.

3. Then, the spores were scrapped using an inoculation needle, under strict aseptic conditions.

4. The spore suspension obtained was adjusted to a concentration of $12 \times 10^8$ spores / ml using sterile physiological saline.

5. The prepared spore suspension was used as the inoculum.
2.2.2.2 Vegetative inoculum

Vegetative mycelial inocula was prepared in 250 ml of GPYS medium (Glucose-1 gm, peptone-0.5 gm, Yeast extract-0.1 gm in one liter sea water, pH-7.6). Prepared medium taken in a one litre flask was inoculated with 10 ml of spore suspension (prepared as described under section 2.2.2.1). The inoculated broth was incubated at room temperature (28 ± 2°C) on a rotary shaker at 150 rpm for 48 hours. The mycelia was collected aseptically by centrifugation at 10,000 rpm for 10 minutes, and washed repeatedly with sterile physiological saline. The separated mycelial pellets were broken down by vigorous agitation with sterile glass beads (3 mm), using a vortex mixer, and suspended in 100 ml of the same saline. The concentration of the prepared suspension was approximately 25 μg dry weight equivalent of mycelia per ml. The prepared suspension was used as vegetative mycelial inoculum.

2.2.3 Inoculation and Incubation

The prepared inoculum was used at 4% (v/v) level (arbitrarily selected before optimisation) and incubated at room temperature (28 ± 2°C), on a rotary shaker at 150 rpm, for 48 hours (unless otherwise specified).

2.2.4 Measurement of growth

Growth was estimated in terms of total protein content of the biomass (Herberts et al., 1971) using Folin's ciocalteu reagent (Lowry et al., 1951), as detailed below.
1. After incubation for the desired period the mycelia were harvested by centrifugation (at 10,000 rpm for 20 minutes, at 4° C),
2. Washed repeatedly (by consecutive centrifugation) with sterile distilled water to remove the residual medium constituents and the metabolites, homogenized with a tissue homogenizer,
3. Suspended in sterile distilled water.
4. 2 ml of the prepared mycelial suspension was taken in a test tube,
5. 2 ml of 1 N NaOH was added
6. The tube with contents was placed in a boiling water bath for 5 minutes.
7. Cooled to room temperature,
8. The undissolved residue was removed by centrifugation at 5000 rpm for 10 minutes.
9. One ml of the supernatant was mixed with freshly prepared 2.5 ml of alkaline reagent (50 ml of 5% Na2CO3 + 2 ml of 0.5% CuSO4 5H2O in 1% sodium potassium tartrate),
10. Allowed to stand for 10 minutes,
11. 0.5 ml of Folin-Ciocalteu reagent was rapidly added
12. Allowed to stand for thirty minutes,
13. The blue colour developed was measured by taking the absorbance at 750 nm in a UV-visible spectrophotometer (Spectronic Genesis, Milton Roy. USA), against the reagent blank.
14. Bovine Serum Albumin was used as standard for computation of protein content and expressed as mg/ml.
15. Protein content was expressed as mg/ml.
2.2.5 Enzyme assay

The fermented broth, after incubation for the desired period, was centrifuged at 10,000 rpm for 20 minutes, at 4°C, in a refrigerated centrifuge (Kubota 6900. Japan) and the cell free supernatant was collected and used for enzyme assay.

L-Glutaminase was assayed according to Imada et al (1973) with slight modifications, as given below.

1. An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of phosphate buffer (0.1M, pH 8.0)

2. The mixture was incubated at 37°C for 15 min. and the reaction was arrested by the addition of 0.5 ml of 1.5 M Trichloro Acetic Acid.

3. To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added.

4. The absorbance was measured at 450nm using a UV-Visible spectrophotometer (Spectronic Genesys5, Milton Roy USA)

5. A standard graph was plotted using ammonium chloride as the standard for computation of the concentration of ammonia, liberated due to enzyme activity

6. One international unit of L-glutaminase was defined as the amount of enzyme that liberates one μ mol. of ammonia under optimum conditions. The enzyme yield was expressed as Units / ml (U/ml)

7. Appropriate controls were included in the experiment.
2.2.6 Determination of Enzyme Protein

The enzyme protein was determined following the method of Lowry et al (1951), as detailed below.
1. To 1 ml of the enzyme, 5ml of alkaline reagent was added.
2. Contents were mixed thoroughly and left for ten minutes.
3. 0.5 ml of Folin's reagent diluted with an equal volume of water was added to each tube.
4. After 40 minutes, absorbance was measured at 750 nm in a UV-Visible Spectrophotometer. (Spectronic Genesys5, Milton Roy USA)
5. Bovine Serum Albumin was used as the standard.
6. Protein was expressed in mg/ml.

2.3 Optimisation of Process parameters for L-glutaminase production under Submerged fermentation (SmF) by marine Beauveria bassiana.

Optimum conditions required for maximum L-glutaminase production under SmF was determined for incubation temperature, pH of the medium, substrate concentration, sodium chloride concentration, additional nitrogen sources, aminoacids, and inoculum concentration, by varying the variables and evaluating the rate of L-glutaminase production.

The protocol adopted for optimization of various process parameters influencing glutaminase production was to evaluate the effect of an individual parameter and to incorporate it at the standardized level in the experiment before optimizing the next
parameter. All the experiments were carried out in triplicate and the mean values are reported.

2.3.1 Temperature

The optimum temperature required for maximal L-glutaminase production by marine Beauveria bassiana under SmF was estimated by incubating the inoculated medium at various temperatures (22, 27, 32, 35, and 42°C) for a period of 48 hours as mentioned under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected and enzyme was assayed as described under section 2.2.5.

2.3.2 pH

Optimal pH required for enhanced level of L-glutaminase production by marine Beauveria bassiana under SmF was determined at various levels of pH (6-13) adjusted in the medium using 1N HCl / NaOH. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.3.3 Additional Nitrogen sources

Requirement for additional nitrogen sources, besides L-glutamine, in the medium for enhanced enzyme production under SmF was determined by incorporating various nitrogen sources (Peptone, Yeast extract, Malt extract, Beef extract, Ammonium sulphate, Ammonium Nitrate, Calcium nitrate and Potassium Nitrate, individually at 1%
(w/v) level in the medium. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the culture broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

Since, yeast extract and Potassium nitrate were found to promote enhanced levels of L-glutaminase production, as additional nitrogen source, optimal concentrations of the same were determined further by incorporating these compounds at different concentrations, (1-5 % w/v) in the medium. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5

2.3.4. Additional Carbon Sources

Need for additional carbon sources, along with glutamine, for enhanced enzyme production by marine Beauveria bassiana under SmF was tested by incorporating maltose, glucose, mannitol, mannose, sucrose and sorbitol, in the medium, individually at 1% (w/v) level. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the culture broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

Since sorbitol, as an additional carbon source, was found to promote enhanced level of enzyme production under SmF, optimal concentration of the same required for the purpose, was determined by incorporating the same at different concentrations (1-7% w/v) in the medium, and evaluating the level of enzyme production. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation,
the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.3.5 **Amino acids**

Impact of amino acids, as inducer substances in the medium, on enzyme production by marine *Beauveria bassiana* under SmF was tested with glutamic acid, asparagine, arginine, methionine, proline and lysine in the medium, at 1 % (w/v) level. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

Since, methionine was found to promote enhanced level of enzyme production, under SmF, optimal concentration of methionine required was determined by evaluating the level of enzyme production at different concentrations of the same (0.1 -1 % w/v) in the medium. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.3.6 **Sodium Chloride concentration**

Impact of sodium chloride on enzyme production by marine *Beauveria bassiana* was determined by subjecting the strain to various levels of NaCl concentration (0-15 % w/v) adjusted in the medium. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.
2.3.7 Time Course Study.

After optimising the various process parameters a time course study was carried out at the optimised conditions. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.4 L-glutaminase production by marine Beauveria bassiana under solid state fermentation (SSF) using inert solid support.

2.4.1 Inert solid support

Expanded polystyrene (poly (1-phenylethylene)), a commercially available insulating and packaging material, was used as inert solid support for solid state fermentation production of L-glutaminase by marine Beauveria bassiana. It is odourless, nontoxic, tasteless, low in weight, less brittle, and non biodegradable. Its maximum water absorbancy is 2.0g/100cm² (Brydson, 1982). Although it is nutritionally inert, it could act as a support for attachment of microorganisms during fermentation (Prabhu and Chandrasekaran, 1995). Moreover, marine microorganisms have capacity to adsorb or anchor onto solid particles. It facilitates the growth of organism. Polystyrene being nutritionally inert, do not support production of undesirable protein in the presence of specific substrate. Whereas, usual SSF media such as wheat bran support synthesis of several undesirable proteins along with the
desired protein. Hence, in the present study polystyrene was used as support for L-glutaminase production by marine Beauveria bassiana under SSF.

2.4.2 Media

L-glutaminase production by Beauveria bassiana under solid state fermentation using polystyrene was optimised using a basal medium containing L-glutamine (10g/L), and D-Glucose(10/g/L) dissolved in aged sea water (35.0 ppt salinity) with pH 8.0.

2.4.3 Preparation of solid substrate medium

Polystyrene beads of 2-3 mm diameter were pretreated by autoclaving at 121°C for 15 min. during which the beads collapsed and reduced to about one third of their original size (Brydson, 1982). The reduced beads of uniform size (1-1.5mm) were used for fermentation studies (Prabhu and Chandrasekaran, 1995).

Ten grams of pretreated polystyrene beads were taken in 250 ml Erlenmeyer flasks, moistened with the prepared medium (as mentioned under section 2.4.2 unless otherwise mentioned), autoclaved for 1 hour and cooled to room temperature before inoculation.

2.4.4 Preparation of Inoculum

Inoculum was prepared as described under section 2.2.2.1
2.4.5 Inoculation and incubation procedures

The sterilized solid substrate media was inoculated with the prepared inoculum (12 x 10^8 spores/ml - arbitrarily selected before optimization of inoculum concentration). Care was taken such that no free water was present after inoculation. The contents were mixed thoroughly and incubated in a slanting position at 27 ± 2°C for 5 days, under 80% relative humidity (Suresh, 1996).

2.4.6 Enzyme recovery

Enzyme, after solid state fermentation using polystyrene, was extracted employing simple contact method using phosphate buffer (0.1M, pH 8.0 (Prabhu, 1996). After mixing the solid fermented substrates with 50 ml of phosphate buffer, the flasks were kept on a rotary shaker (150 rpm) for 30 minutes, and the contents were pressed in a dampened cheese cloth to recover leachate. The process was repeated twice, the extracts were pooled, and centrifuged at 10,000 rpm for 20 minutes at 4°C in a refrigerated centrifuge. The supernatant was used for the enzyme assay.

2.4.7 Assays

2.4.7.1 L-Glutaminase

L-Glutaminase was assayed according to Imada et al (1973), with slight modifications, as described under section 2.2.5.
2.4.7.2 Protein

Total cell protein and enzyme protein were estimated using Lowry's method with bovine serum albumin as the standard (Lowry et al., 1951), as described under sections 2.2.4 and 2.2.6, respectively. The values were expressed as mg/ml.

2.5. Optimization of process parameters for L-glutaminase production by marine *Beauveria bassiana* under solid state fermentation using polystyrene as inert support.

The impact of various process parameters on L-glutaminase production by marine *B. bassiana* under SSF using polystyrene as inert support was evaluated. The various process parameters studied include incubation temperature, initial pH of the medium, initial moisture content of the medium, inoculum concentration (both spore and vegetative), sea water concentration, substrate (L-glutamine) concentration, additional NaCl concentration, additional carbon source, additional nitrogen source, and amino acids. Finally a time course study was carried out incorporating all the optimised parameters.

The protocol adopted for optimisation of various process parameters influencing glutaminase production was to evaluate the effect of an individual parameter and to incorporate the same at the standardized level in the subsequent experiment before optimizing the next parameter (Sandya and Lonsane, 1994). All the experiments were carried out in triplicate and the mean values are reported.
2.5.1. Initial pH of the medium

The effect of initial pH of the sea water based medium on enzyme production by *B. bassiana* during SSF was studied at various pH levels (pH 4-11) adjusted in the enzyme production medium using 1N NaOH / 1N HCl. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.2. Incubation temperature

Optimal temperature required for maximal enzyme production by *B. bassiana* under SSF was determined by incubating the inoculated flasks at different temperatures (22-47°C) under 75-80% relative humidity. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.3. Additional carbon sources

Requirement for additional carbon sources other than L-glutamine, in the medium, for enhanced enzyme production by *B. bassiana* under SSF was tested by the addition of different carbon sources (glucose, maltose, lactose, Sucrose, sorbitol and mannitol) at 1% (w/v) level in the SSF medium. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.
2.5.3.1. D-glucose

Since D-glucose was found to promote enhanced enzyme production (2.4.8.3) as additional carbon source optimal concentration of the same, was determined by conducting the solid state fermentation at different concentrations of the same (0.25-4.0 % w/v) incorporated in to the SSF medium. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.4. Additional nitrogen sources

Requirement for additional nitrogen sources, other than L-glutamine, for enhanced enzyme production by \textit{B. bassiana} under SSF was tested by the addition of different nitrogen sources (Beef extract, malt extract, yeast extract, peptone, ammonium sulphate, ammonium nitrate, calcium nitrate and potassium nitrate) at 1% (w/v) level in the SSF medium. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.5. Amino acids

Requirement for amino acids in addition to L-glutamine for enhanced enzyme production by \textit{B. bassiana} was tested by the addition of different amino acids (glutamic acid, asparagine, arginine, methionine, proline and lysine,) at 1% (w/v) level in the SSF medium. Preparation of solid substrate, preparation of inoculum, inoculation and
incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

**2.5.6. L-glutamine concentration.**

The influence of L-glutamine concentration on glutaminase production by *Beauveria bassiana* during SSF was evaluated by supplementing the fermentation medium with different concentrations of glutamine (0.1-5.0% w/v). Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

**2.5.7. Initial moisture content.**

Optimal level of initial moisture content of the solid support medium required for maximal enzyme production under SSF was determined by preparing the solid substrates with varying levels of moisture content in the range of 60-100%, using different quantities of the basal media prepared in sea water (section 2.4.2). Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

**2.5.8. Inoculum concentration**

**2.5.8.1. Spore inoculum**

Optimal concentration of spore inoculum that enhance maximal enzyme production under SSF by *B. bassiana* was determined at different spore concentrations ranging from
6.0 - 48 x 10^8 spores/ml. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.2.2.1, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.8.1. Vegetative inoculum

Optimal concentration of vegetative mycelial inoculum that enhance maximal enzyme production under SSF by *B. bassiana* was determined at different inoculum concentrations varying from 0.125 - 0.750 mg/ml (w/v). Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.2.2.2, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.9. Sea water concentration

The optimal concentration of aged sea water required for enhanced glutaminase production by *B. bassiana* during solid state fermentation was evaluated by diluting with distilled water to various levels of concentration (0, 25, 50, 75 and 100% v/v) and preparing the solid state fermentation media. Aged sea water as such was considered as 100% for comparison purposes. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.
2.5.10. Additional NaCl Concentration

Effect of NaCl, added to aged sea water, on enzyme production of B. bassiana under solid state fermentation was evaluated by incorporating NaCl at different concentrations (0.0 to 4.0% w/v) in the medium, prepared with aged sea water. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.11. Time course study

After optimising the various process parameters, a time course study was carried out by providing optimised bioprocess conditions. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively. Total cell protein and Enzyme protein were also determined as described under 2.2.4 and 2.2.6, respectively.

2.6. L-glutaminase production by terrestrial Beauveria bassiana under solid state fermentation and submerged fermentation.

L - glutaminase production by terrestrial B. bassiana NCIM 1216 (obtained from the culture collection of National Chemical Laboratory, Pune, India) was determined under solid state fermentation and submerged fermentation conditions using media based on both sea water and distilled water at enzyme production conditions optimised for the marine Beauveria bassiana strain.
For solid state fermentation preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.2.2.1, 2.4.5, 2.4.6 and 2.2.5 respectively.

For submerged fermentation, inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.7. L-glutaminase production by immobilized marine Beauveria bassiana.

Although several methods are available to effect immobilization such as entrapment, cross linking, covalent binding and physical adsorption, in the present study gel entrapment method using calcium alginate was adopted for the preparation of immobilized spores of marine B. bassiana.

2.7.1. Media

Enzyme production media used for L-glutaminase production by immobilized spores of marine B. bassiana included:

- L-glutamine: 0.25 %
- D-glucose: 0.5 %
- pH: 9.0
- Aged sea water: 100 ml.

The prepared medium was autoclaved at 121°C for 1 hour and used.
2.7.2 Preparation of spore suspension

Spore suspension was prepared as described under section 2.2.2.1

2.7.3 Preparation of support material for immobilization

Initially 6g of sodium alginate was slowly added to 150 ml of distilled water (4%) while being continuously stirred (Mohandass, 1992). The stirring was continued for a further period of 1-2 hours in a magnetic stirrer until complete dissolution of the sodium alginate was effected. The prepared sodium alginate solution was autoclaved at 121 °C for 20 minutes and used for preparation of beads.

2.7.4 Preparation of beads

Beads with immobilized fungal spores were prepared as suggested by Mohandass, (1992). Under sterile conditions the prepared spore slurry was mixed with the sodium alginate solution, at a concentration of $12 \times 10^8$ spores/ml, at a ratio of 1:2 and mixed thoroughly using a sterile glass rod to get a spore- alginate slurry. This was then extruded drop wise in to a solution of 0.2 M CaCl$_2$ from a height of about 10 cm using a syringe with a pore size of 2 mm. The entrapped calcium alginate beads were then maintained in a solution of CaCl$_2$ (0.2 M) for 2 hours for curing. After this the beads were thoroughly washed with physiological saline 3-4 times and maintained at 4°C until use.
2.7.5. Activation of immobilized spores.

The prepared beads with immobilized fungal spores were suspended in a solution of 1% glutamine in aged sea water, pH 9.0, in a conical flask and incubated on a rotary shaker (90 rpm), at 28 ± 2 °C, for 14 hours (prior to optimisation of activation time). The activated beads were then removed, washed twice with fresh enzyme production medium and used for further studies.

2.7.6. Incubation procedures

Twenty grams of beads with immobilized spores were weighed, transferred to 50 ml of enzyme production media taken in a 250 ml conical flask, kept on a rotary shaker (90 rpm), and incubated for 24 hours at room temperature (28 ± 2 °C).

2.7.7. Enzyme assay

After incubation for the desired period, the enzyme production media was decanted from the flask, centrifuged at 10,000 rpm for 10 minutes at 4 °C in order to remove leached out spores if any. Supernatant obtained was assayed for L-glutaminase following the method of Imada et al (1973), with slight modifications as described under section 2.2.5.

2.8. Optimisation of immobilization process conditions.

Optimal concentration of immobilizing support material, concentration of fungal spore in the beads, CaCl₂ concentration, curing time of beads, activation time and
retention time, incubation temperature and pH of the media, that could promote maximal production of L-glutaminase by immobilized *B. bassiana* were determined as detailed below.

### 2.8.1 Support concentration

The optimal support concentration required for the preparation of active and stable beads with maximum enzyme production was determined using sodium alginate at different concentrations (1.5-5% w/v). Immobilized spore in beads were prepared as mentioned under section 2.7.4.

### 2.8.2 Spore concentration in the beads

Beads with immobilized spore were prepared using spores at different concentrations (2-16x10^8 spores/gm beads) and effect of the same on L-glutaminase production was determined (section 2.7.7).

### 2.8.3 Calcium Chloride concentration

The optimal concentration of calcium chloride required for the preparation of active and stable immobilized viable spore beads with maximum enzyme production was determined by using calcium chloride at different molar concentrations (0.05M - 0.4 M).

### 2.8.4 Curing time

Optimum curing time for preparing stable immobilized viable spore beads were determined by allowing the beads formed in 0.2 M CaCl\_2 to remain as such for varying periods of curing time (1-5 hours). Later, the beads were washed with physiological
saline and the optimal curing time was assessed by estimating L-glutaminase (section 2.7.7) production in the medium.

2.8.5 Activation Time

Optimal activation time for enhanced L-glutaminase production by immobilized fungal spores was determined by incubating the beads with immobilized spores in a solution of 1 % glutamine in sea water, pH 9, on a rotary shaker at 90 rpm for varying time intervals (5-25 hours) at room temperature (28 ± 2°C), followed by incubation in enzyme production medium as described under section 2.7.6. The optimal activation time was assessed by quantifying the L-glutaminase (section 2.7.7) in the media.

2.8.6 Retention time

Optimal retention time required for maximal L-glutaminase production was determined by incubating the beads with immobilized fungal spores in the production medium for varying periods and estimating L-glutaminase production as mentioned under section 2.2.5.

2.8.7 Incubation Temperature

Optimal temperature that support the maximum enzyme production by immobilized spores of *B. bassiana* was found out by incubating the beads at different
temperatures 22, 27, 32, and 37°C and then estimating the L-glutaminase production as mentioned under section 2.7.7

2.8.8. pH of the media

Optimal pH of the media that support maximal enzyme production by beads with immobilized spores was determined by subjecting the beads to media with different pH, ranging from 5-10, and then estimating the L-glutaminase production as mentioned under 2.7.7

2.9. Continuous production of L-glutaminase by immobilized spores of marine B. bassiana.

Continuous production of L-glutaminase by gel entrapped immobilized spores of marine B. bassiana was monitored in a Packed Bed Reactor. The effect of flow rate of the media into the reactor, aeration rate and bed height, were determined. The cumulative production of glutamine in packed bed reactor was also determined.

2.9.1. Packed Bed Reactor.

The cylindrical glass column of 5 cm diameter, 30 cm length was used as the reactor (Fig.1). The bottom of the column which was hemispherical in shape with a 5 mm diameter inlet, was packed with small amount of glass wool and glass beads of 4 mm diameter up to 20 mm height. A sieve plate with 1 mm perforations were placed over the glass beads and immobilized spore beads were packed up to different heights. A sieve plate was placed over the beads and fixed to the top of the reactor. The column
Fig. 1

Continuous production of glutaminase in a packed bed reactor

Experimental set up for packed bed reactor studies
was provided with a side tube of 5 mm diameter at the top portion, through which the sample broth was withdrawn. The reactor was fed from the bottom using a peristaltic pump (Murhopye, India Ltd.) and the effluent was removed from the top. Sterile air was admitted into the reactor through a bacterial filter (Millipore) from an air pump with flow meter (Eyela, Japan ).

2.9.2 Activation of the packed bed reactor

The immobilized bed in packed bed reactor was activated using 1% glutamine solution in sea water (pH 9) for a period of 15 hours, before commencing the experiment.

2.9.3 Estimation of void volume

The voidage of the packed bed reactor was determined by measuring the volume of the liquid actually occupied in the reactor. The reactor was packed with spherical gel beads to the desired level and bed depth was noted. Physiological saline was filled from the bottom with the help of the peristaltic pump till the liquid layer reached to the top layer of the gel beads. The liquid was then slowly withdrawn from the column till its level reached the bottom layer of the bead and the quantity of saline thus collected was measured. The procedure was repeated thrice and the average value was taken.

2.9.4 Enzyme Recovery

Effluent medium, from the packed bed reactor, was recovered from the upper outlet of the reactor at one hour intervals and those along with the accumulated effluent
from the container, was centrifuged at 10,000 rpm, at 4°C for 20 minutes and the supernatant was used for enzyme assay.

2.9.5. Enzyme assay

L-Glutaminase in the effluent medium collected from the outlet and container were assayed separately following the method of Imada et al (1973) with slight modifications, as described under section 2.2.5.

2.9.6. Flow rate

Effect of flow rate of the media on the continuous production of L-glutaminase by the immobilised spores of marine Beauveria bassiana and the performance of the immobilised reactor was evaluated by monitoring the rate of L-glutaminase production under different flow rates (20-60 ml/hour). For each flow rate samples were withdrawn after one hour interval for up to five hours and the rate of enzyme production was determined.

2.9.7. Substrate concentration

Optimal substrate concentration required for maximal glutaminase production by immobilized spores of B.bassiana under continuous production in a packed bed reactor was determined as detailed below. Prepared beads of immobilized spores were packed to the required height in the reactor and the substrate solution having different concentrations (0.125 - 1.0 %) were passed through the reactor one after another separate cycles. Enzyme production was assayed as described under section 2.2.5.
2.9.8. Aeration rate

The effect of aeration on L-glutaminase production by immobilized *B. bassiana* was determined by supplying sterile air through a filter at rates ranging from 0.4 - 1.7 vvm.

2.9.9. Bed Height

Optimal bed height required for maximal glutaminase production by immobilized *Beauveria bassiana* under continuous production in a packed bed reactor was determined by raising the bed height from 5 - 15 cms. at a flow rate of 20 ml/hr. at 28 ± 1°C. Enzyme production was assayed as described under section 2.2.5.

2.10. Statistical analysis.

All the experiments and analysis were carried out in triplicate and mean values alone are taken. Statistical Analysis (Standard Deviation) was done using SIGMA STAT- version 2.01