Optimum conditions for L-glutaminase production by actinomycete strain isolated from estuarine fish, *Chanos chanos* (Forskal, 1775)

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Actinomycetes were isolated from skin, gills and gut contents of estuarine fish, *Chanos chanos* using Kuster’s agar medium. Out of 20 strains tested, the strain LG-10 which was tentatively identified as *Streptomyces rimosus* showed L-glutaminase activity. Optimum production of L-glutaminase enzyme (17.51 IU/ml) was observed after 96 h of incubation at 27°C, pH 9 and glucose and malt extract as carbon and nitrogen sources, respectively. The present study indicated scope for the use of *S. rimosus* as an ideal organism for the industrial production of extracellular L-glutaminase.

**Keywords:** Actinomycetes, *Chanos chanos*, Estuarine fish, L-Glutaminase, *Streptomyces rimosus*

The enzyme, L-glutaminase (L-glutamine amidohydrolase EC 3.5.1.2) has attracted much attention due to its application in both pharmaceutical and food industries. L-glutaminase in combination with or as an alternative to L-asparaginase could be of significance in enzyme therapy of cancer, especially acute lymphocytic leukemia. It is well known that most of the basic flavour components of fermented condiments are amino acids produced by the enzymatic degradation of proteins contained in the raw materials and among them, L-glutamic acid is a widely acclaimed flavour-enhancing amino acid. For example, the unique flavour of fermented soy sauce is attributed mainly to glutamic acid (concentration at 0.7 to 0.8% per total nitrogen). Activity of L-glutaminase, which is responsible for synthesis of glutamic acid, makes it an important additive during soy sauce fermentation. Bacteria and fungi are the potential candidates for production of L-glutaminase enzyme. In the recent years, L-glutaminase enzyme is produced on a large scale using recombinant *E. coli* and sold at higher price. From the available literature, it is found that information on L-glutaminase production from marine actinomycetes is scanty. Therefore, the present study was designed to explore actinomycetes from fish, to optimize the production of L-glutaminase enzyme from these actinomycetes and also to identify the potential ones using conventional and chemotaxonomical methods of identification.

A total of 20 actinomycetes strains were isolated from various body parts of the fish, *Chanos chanos* (Forskal, 1775) collected from the Vellar estuary (Lat. 11° 29’N, Long. 79° 46’E) and were used for the present study. All chemicals collected from Himedia Laboratories Pvt. Ltd., Mumbai, were used for carrying out the experiments. Actinomycetes isolated from various parts of fish viz., *C. chanos* using Kuster’s agar medium were screened for L-glutaminase activity. The packed cells were suspended in distilled water and inoculated in 5 ml of glycerol-glutamine broth and incubated for seven days at 30°C. After day 7, the broth was filtered through filter paper (Whatman number 1) and activity of L-glutaminase was measured by adding Nessler’s (0.5 ml) reagent to the filtered culture broth. Within 5 min, a yellow colour was developed. Then the sample was centrifuged at 27,000 rpm for 10 min and absorbance of the supernatant was read at 450 nm² using a UV-visible spectrophotometer (Hitachi). Ammonia content was estimated using standard ammonium chloride solution and protein content of the enzyme preparation was estimated following the method of Lowry et al.³. L-glutaminase activity has been expressed in International Units per mg of protein (IU). One IU is the amount of enzyme required to liberate 1 μmol of ammonium in 1 min under experimental conditions. Ammonium sulphate was used as standard.

Factors influencing the secretion of L-glutaminase enzyme like temperature, pH, different carbon compounds and nitrogen compounds, various concentrations of sodium chloride and incubation periods were optimized by a single factor of varying the parameters one at a time. Experiments were conducted in Erlenmeyer flasks (250 ml) containing glycerol-glutamine broth. After sterilization of the broth by autoclaving at 121°C under 15 lbs pressure for 15 min, the flasks

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were cooled and the strains were inoculated (5 mg/ml) and incubated for 7 days (except for incubation period experiment) separately as described. Fermentation was carried out in triplicate at different temperatures (21°, 24°, 27°, 30°, 33° and 36°C), pH (5, 6, 7, 8, 9 and 10), carbon sources (1% of glucose, maltose, mannitol, mannoce, sucrose and sorbitol), nitrogen sources (1% of peptone, yeast extract, beef extract, malt extract, calcium nitrate and potassium nitrate), sodium chloride concentrations (0, 1, 2, 3, 4 and 5%) and incubation periods (24, 48, 72, 96, 120 and 144 h). Optimum condition identified for one parameter was used for optimizing the other parameters one by one.

The genus level identification was made for the strain LG-10 which showed good enzymatic activity using cell wall composition analysis and micromorphological studies. Characterization of the strain was made by following the methods described by Shirling and Gottlieb. The species level identification of the strain was made based on the keys of and Bergey’s Manual of Determinative Bacteriology. Subcultured strain in multiple was maintained at -20°C.

As it is very difficult to obtain sufficient quantity of L-glutaminase from marine microorganisms, not many studies on this enzyme have been carried out. Except for the studies of and on L-glutaminase from Beauveria sp. and Beauveria bassiana isolated from the marine sediments of Cochin, Kerala, no other literature is available from Indian coastal regions. In the present study, out of 20 actinomycetes strains tested, only one strain (LG-10) showed good L-glutaminase activity and it produced L-glutaminase extracellularly in a broth containing 1% (w/v) L-glutamine after 48 h before optimization of process parameters. Subsequently, various process parameters which influence L-glutaminase production were optimized (Table 1). Initial pH of the medium, at 27°C, influenced L-glutaminase production. Two pH optima were observed that favoured high enzyme yield, one at pH 6 (13.98 IU/ml) and another at pH 9 (14.74 IU/ml) (Fig. 1). 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. Incubation at 27°C, at pH 9 (optimized), enhanced enzyme production (14.74 IU/ml) as compared to other temperatures. But, a considerable level of enzyme production could be obtained at other pH and temperature also. These factors are characteristics of the organisms and vary from species to species.

Supplementation of the media with different carbon sources enhanced the enzyme yield from 8.19 to 15.11 IU/ml. Among the various carbon sources tested, glucose gave maximal yield (15.11 IU/ml) compared to others. This was in conformity with the findings of Prabhu and Chandrasekaran on the marine Vibrio costicola under solid state fermentation. Such enhanced production of L-glutaminase by supplementation of carbon sources could be attributed to the positive influence of additional carbon sources on the enhanced biosynthesis. Nitrogen can be an important limiting factor in the microbial production of enzymes, among the different nitrogen sources tested, malt extract yielded higher enzyme production (15.61 IU/ml). Presence of nitrogen source along with L-glutamine in the medium has probably promoted growth and consequent enzyme production.

L-glutaminase production was maximum (13.59 IU/ml) at 2% of NaCl concentration. Addition of NaCl to the medium led to a decline in the enzyme production. This would indicate that the strain LG-10 is not halophilic, but could be halotolerant and a natural commensal organism in the marine environment. Further, it is understood that the sea water could be an

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>21</th>
<th>24</th>
<th>27</th>
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<tr>
<td>Enzyme activity (IU/ml)</td>
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<td>13.05</td>
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<td>13.58</td>
<td>13.98</td>
<td>14.05</td>
<td>14.52</td>
<td>14.74</td>
<td>14.61</td>
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<tr>
<td>Carbon sources (1%)</td>
<td>Glucose</td>
<td>Maltose</td>
<td>Mannitol</td>
<td>Mannose</td>
<td>Sucrose</td>
<td>Sorbitol</td>
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<tr>
<td>Enzyme activity (IU/ml)</td>
<td>15.11</td>
<td>11.57</td>
<td>12.69</td>
<td>8.19</td>
<td>9.53</td>
<td>9.87</td>
</tr>
<tr>
<td>Nitrogen sources (1%)</td>
<td>Peptone</td>
<td>Yeast extract</td>
<td>Beef extract</td>
<td>Malt extract</td>
<td>Calcium nitrate</td>
<td>Potassium nitrate</td>
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<td>15.03</td>
<td>15.27</td>
<td>15.61</td>
<td>14.93</td>
<td>14.51</td>
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<td>NaCl concentration (%)</td>
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<td>4</td>
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<tr>
<td>Enzyme activity (IU/ml)</td>
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<td>13.59</td>
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<td>9.28</td>
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<td>12.08</td>
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ideal medium or at least adequate to support L-glutaminase synthesis. With regard to the different incubation periods, the maximum L-glutaminase production (17.51 IU/ml) was observed after 96 h of incubation, as has been also observed in *Beauveria* sp. and *B. bassiana*\(^8\). The time course study after process optimization, showed higher enzyme production (17.51 IU/ml) after 96 h of incubation at pH 9 and 27°C when glucose and malt-extract were used as carbon and nitrogen sources respectively.

The strain LG-10 possesses LL-diaminopimelic (LL-DAP) and it contains glycine in its cell wall. Presence of LL-DAP along with glycine indicates the cell wall chemotype – I (Ref. 4), which is the characteristic of the genera, *Streptomyces*, *Streptovercicillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elytrosporangium*, *Microellobosporia*, *Sporichthya* and *Intrasporangium*\(^4\). The micromorphological properties of the strain LG-10 are similar to the genus *Streptomyces*. Hence, the morphological, physiological and biochemical characteristics of the strain LG-10, were compared with those of the *Streptomyces* species given in the key of Nonomura\(^6\) and those species described in the Bergey’s Manual of Determinative Bacteriology\(^7\). The strain LG-10 showed close similarity with the reference strain *Streptomyces rimosus* (Table 2) when compared and varied only in utilization of carbon compounds viz., rhamnose and sucrose. Except this, all other characters were similar to those of *S. rimosus*. Hence, the strain LG-10 was tentatively identified as *S. rimosus*. Thus, the present study indicated that the strain LG-10 *S. rimosus* had the potential for the industrial production of extracellular L-glutaminase. Further studies in detail are required for commercial use of the strain for production of L-glutaminase.

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### References


