ISOLATION AND PRODUCTION OF L-ASPARAGINASE FROM SOIL CONTAINING COLIFORM BACTERIA

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Abstract

L-asparaginase can be effectively used for the treatment of acute lymphoblastic leukemia and tumour cells. For pharmacological and clinical tests, microbial sources are best for the bulk production of L-asparaginase. A reported work is mainly focus on the production of L-asparaginase from coliform bacteria as they are involved in an elaboration of L-asparaginase. Coliform bacteria were isolated from the soil samples which were collected from the various places of Guntur by using modified ISP-5 medium and multiple tubes lactose fermentation method. Of the 12 isolates AS-2 strain was found to be the Citrobacter sp. and also involved in an elaboration of L-asparaginase. It was characterized by taxonomical and morphological studies. The effect of carbon and nitrogen sources on the production of L-asparaginase was investigated. Glucose at 1.0% and Yeast extract at 1.5% exhibited maximum L-asparaginase production. The other parameters such as pH, temperature and incubation period were optimized and set at 8, 37°C and 26 h, respectively. L-asparaginase has been partially purified with ammonium sulphate (20-40%)

Introduction

L-Asparaginase (L-asparagine amino hydrolase, E.C. 3.5.1.1, LA) catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. As the several types of tumour cells require L-asparagine is an essential amino acid for protein synthesis; they
are deprived of an essential growth factor in the presence of LA. Effective depletion of L-asparagine results in cytotoxicity for leukemic cells.

Aim and objectives

Among the bacteria, Coliform bacteria have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions. To explore the deriving new products of economic importance. The reported work deals with isolation of coliform bacteria and optimization of various parameters to get the maximum yield of L-asparaginase.

Materials and methods

Collection of Soil Samples

Soil samples were collected from different places of Guntur, Andhra Pradesh, India. The reported work was carried out in Department of Biotechnology, Vagdevi College of Pharmacy, Gurazala, Andhra Pradesh, India. Soil is collected in sterile bag. Dried soils surface sterilized by using tween80. Then soils are serially diluted from $10^{-1}$ to $10^{-9}$ by using sterile water.

Screening of Coliform Bacteria Serial Tube Method

MacConkey broth was sterilized (autoclaved at 120°C for 20 min), distributed equally (5 ml) in each test tubes. All the tubes were inoculated with 1ml of soil sample from $10^{-1}$ to $10^{-9}$ and kept at 37°C for overnight incubation. Presence of coliform was further characterized by streaking from positive MacConkey broth tubes to Eosin-Methylene Blue agar.

Modified ISP-5 Medium (Rapid Screening Method)

Modified ISP-5 medium (L-asparagine 1 g, Dipotassium phosphate 1 g, trace salts solution 1 ml, lactose 20 g, phenol red 1 ml, agar 20 g, pH 7.0) was sterilized (autoclaved
at 120°C for 20 min), inoculated with 1 ml of collected soil samples and poured to the Petri plates. All the plates were incubated at room temperature including the control plates, which were without carbon source (lactose).

**Identification of Isolates**

Isolated colonies were identified through the lactose fermentation test, Gram staining and biochemical tests such as H₂S production, indole production, MR-VP test, citrate utilization test etc. (Cappuccino and Shearman, 2006; Sleigh and Duguid, 2005). The isolated were screened for asparaginase activity as per the direct nesslerization method. The TLC was performed to check the conversion of L-asparagine into L-aspartic acid. Mobile phase: n-butanol: acetic acid: water (5:4:1) and ninhydrine as detecting agent.

**Optimization of Fermentation Parameters**

Both the environmental and nutritional parameters were optimized by using TGY (Tryptone glucose yeast extract) broth as a Basal medium; Glucose 1 g, Dipotassium hydrogen phosphate 1 g, Yeast extracts 5 g, Tryptone 5 g, pH 7.0 (Peterson and Ciegler, 1969) in lab scale fermentor (Sartorius B-lite, Bangalore). Enzyme activities were determined at regular intervals of 12 up to 48 h.

**Optimization of Environmental Parameters Optimization of pH**

The effect of pH on L-asparaginase production was studied by growing AS-2 strain in basal medium of different pH (5, 6, 7, 8, 9 and 10). The pH was maintained by using phosphate buffer.
Optimal temperature for the productivity of AS-2 strain was determined by keeping the inoculated basal media at 27 and 37°C separately.

**Optimization of Nutritional Parameters Optimization of Carbon Source**

The basal medium contains 0.1% of glucose as carbon source. In this study, optimization was done using Glucose and Sucrose with concentrations of 0.1, 0.5, 1.0 and 1.5%, separately. Basal medium with these different carbon sources with their individual concentrations were sterilized and inoculated with AS-2 strain; incubated at 37°C for 48 h.

**Optimization of Nitrogen Source**

The basal medium contains 0.5% of yeast extract as nitrogen source. In this study, optimization was done using Peptone and yeast extract in individual concentrations of 0.1, 0.5, 1.0 and 1.5%. Basal medium with these nitrogen sources were sterilized and inoculated with AS-2 strain; incubated at 37°C for 48 h.

**Determination of Biomass Production**

Optimal duration for the enzyme production was measured by using optimized fermentation parameters. The AS-2 strain was inoculated in optimized TGY broth and incubated for 40 h. Samples were withdrawn at every hour. Biomass production was measured by dry weight method.

**Partial Purification of L-asparaginase**

L-asparaginase was partially purified by the method described by Bilimoria (1969). Solid (NH₄)₂SO₄ was added to the supernatant fluid to achieve 20% saturation. The suspension was further centrifuged and solid (NH₄)₂SO₄ was added to supernatant to
reach 40 and 60% saturation. The precipitates (0-20, 20-40 and 40-60%) were collected by centrifugation. The collected precipitates were dissolved in Tris-HCL buffer, pH 8.0 and dialyzed overnight against the same buffer. After the exclusion of ammonium sulphate, enzymatic activity in each dialyzed solution was determined by nesslerization method. The fraction showing the highest enzyme activity was designated as Partially Purified Extract (PPE).

Results and discussion

Two different methods used for the isolation of coliform bacteria mentioned.

In multiple tubes lactose fermentation after the incubation period, we observed the change in color of MacConkey broth initially red to yellow due to the fermentation of lactose into the acidic metabolites by coliform bacteria which were differentiated from non-coliform as well as other gram negative bacteria by streaking EMB agar from previously positive MacConkey broth tubes. By this method we have screened two bacteria coded as AS-1 and AS-2.

Fig. ISP-5 Medium with lactose after 7 days of incubation
In another method developed in our laboratory in which we modified the original ISP-5 medium. Which contains L-asparagine, a substrate for the L-asparaginase and original carbon source i.e., glycerol was replaced with lactose. Phenol red was incorporated in the medium as pH indicator. Modified ISP-5 medium without lactose was served as control to observe whether the medium color changes from yellow to pink due to the lactose fermentation or the conversion of L-asparagine into the L-aspartic acid and ammonia. We observed high intensity pinkish colour after 2 days incubation and low intensity color after the 7 days incubation in modified ISP-5 medium with lactose and without lactose, respectively (Fig. 1, 2). From this we concluded that pinkish color surrounding the bacterial colonies was due to the liberation of ammonia which makes the pH of the medium alkaline. Colonies with the pinkish surrounding were coded as AS-3, AS-4, AS-5, AS-6, AS-7, AS-8, AS-9, AS-10, AS-11 and AS-12 (Fig. 3).

All isolates were firstly subjected to the lactose fermentation and gas production to satisfy the definition of coliform bacteria. Table 1 shows the result of lactose fermentation and gas production.
Table 1: Test for lactose fermentation and gas production

<table>
<thead>
<tr>
<th>Isolates</th>
<th>AS-1</th>
<th>AS-2</th>
<th>AS-3</th>
<th>AS-4</th>
<th>AS-5</th>
<th>AS-6</th>
<th>AS-7</th>
<th>AS-8</th>
<th>AS-9</th>
<th>AS-10</th>
<th>AS-11</th>
<th>AS-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose fermentation</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gas production</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

+: Positive reaction, -: Negative reaction

Table 2: Biochemical tests

<table>
<thead>
<tr>
<th>Isolates</th>
<th>AS-1</th>
<th>AS-2</th>
<th>AS-7</th>
<th>AS-9</th>
<th>AS-10</th>
<th>AS-11</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2S</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acid from lactose</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MR</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>VP</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Ind</td>
<td>+</td>
<td>+</td>
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<td>Pad</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Cit</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Arg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Ure</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gram staining</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Morinity</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+: Positive reaction; -: Negative reaction; ++: Strong positive reaction; H2S: Hydrogen sulfide; MR: Methyl red; VP: Voges-Proskauer; Ind: Indole; Pad: Phenylalanine deaminase; Cit: Citrate utilization; Arg: Arginine decarboxylase; Ure: Urease; -Ve: Gram negative

For the further characterization we selected isolates which were either lactose fermenting or gas producing or both. Table 2 shows the results of various biochemical tests carried out to identify the lactose fermenting and gas producing isolates.
From the identification studies the AS-2 strain was found to be Gram -ve, rod shaped and lactose fermenting, also producing H$_2$S, utilized citrate as carbon source and Voges-Proskauer negative. All the characters of AS-2 strain were compared with the characters of Enterobacteriaceae family described by Sleigh and Duguid (2005) which help us to conclude that the AS-2 was closely related to *Citrobacter* sp. Before the optimization of fermentation parameters, AS-2 strain was checked for enzyme production by growing it into TGY broth, which showed positive result. The TLC was performed to check the conversion of L-asparagine into L-aspartic acid. The results showed that an Rf value of sample i.e., 0.68 almost the same to that of standard L-aspartic acid i.e., 0.70.

Fig. 4: Optimization of pH

![Optimization of pH](image)

Fig. 5: Optimization of temperature

![Optimization of temperature](image)

As the prime requirement of the biotechnological processes is high yielding organisms, to satisfy this requirement preliminary optimization of various
fermentation parameters are necessary. For this purpose we optimized various environmental and nutritional parameters. The extra cellular pH has a strong influence on the pathways of metabolism and product generation by micro-organism and optimum temperature is also important as it affects the conversion efficiency of substrate into cell mass which affect the product formation, particularly when product is growth associated. So in this study, we optimized pH and temperature as environmental parameters for increasing the L-asparaginase yield. Our studies indicated that pH-8 is optimum extra cellular pH with the enzymatic activity (810 IU mL\(^{-1}\)) at 24 h (Fig. 4). The 37°C found to be an optimal temperature for the L-asparaginase production with enzymatic activity (658 IU mL\(^{-1}\)) at 24 h (Fig. 5).

The components of the fermentation medium should be supplied in an adequate quantity for growth, energy and building of cellular components and synthesis of fermented products where carbon and nitrogen sources play an important role. For this purpose we optimized carbon and nitrogen sources as nutritional parameters. The present studies stated that 1.0% glucose in basal medium is required for an optimal production of L-asparaginase with a yield of 650 IU mL\(^{-1}\) (Fig. 6) whereas 1.0% sucrose showed less yield (361 IU mL\(^{-1}\)) at 24 h (Fig. 7). Different nitrogen sources such as peptone and yeast extracts were used for the production of maximum L-asparaginase and the results showed that yeast extracts with 1.5% concentration in basal medium gave maximum enzyme production with a yield of 371 IU mL\(^{-1}\) (Fig. 8) and 1.0% peptone having a lower yield of 315 IU mL\(^{-1}\) at 24 h (Fig. 9).
Fig. 6: Optimization of glucose

Fig. 7: Optimization of sucrose

Fig. 8: Optimization of yeast extract
By using the optimized fermentation parameters optimal study was determined, results showed that enzymatic activity was at lowest values in the log phase and increasing in the exponential phase; at 26 h it reached to the maximum values and in the early stationary phase (up to 30 h) of the growth cycle the activity was stable and continued in decreasing at late stationary phase. The results stated that production of L-asparaginase was growth associated (Fig. 10). As the L-asparaginase is intracellular product, in the purification studies cells were separated by centrifugation and supernatant was discarded. A summary of purification steps is shown in Table 3. It is clear that the enzymatic activity in the protein fraction 20-40% represented 2.15 purification fold with 20% yields and specific activity of 82 IU mL$^{-1}$. 

Table 3: Partial purification of L-asparaginase

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein* (mg mL$^{-1}$)</th>
<th>Total activity (IU mL$^{-1}$)</th>
<th>Specific activity (IU ml$^{-1}$)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruptured cells</td>
<td>0.450</td>
<td>4.0</td>
<td>38</td>
<td>100.0</td>
<td>1.00</td>
</tr>
<tr>
<td>MnCl$_2$ extract</td>
<td>0.380</td>
<td>3.1</td>
<td>46</td>
<td>77.5</td>
<td>1.21</td>
</tr>
<tr>
<td>0-20%</td>
<td>0.151</td>
<td>2.0</td>
<td>66</td>
<td>50.0</td>
<td>1.73</td>
</tr>
<tr>
<td>20-40%</td>
<td>0.90</td>
<td>0.8</td>
<td>82</td>
<td>20.0</td>
<td>2.15</td>
</tr>
</tbody>
</table>

*Protein was determined by method of Lowry et al. (1951)