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# Screening for L - Asparaginase Producing Actinomycetes from Western Ghats of Karnataka, India

KEYWORDS: Actinomycetes, Western Ghats, L-asparaginase, Streptomyces

**Akshatha. M. D**
Department of Biotechnology, The Oxford College of Engineering, Hosur road, Bommanahalli, Bengaluru-560068

**Manjunatha. B. K**
Department of Biotechnology, The Oxford College of Engineering, Hosur road, Bommanahalli, Bengaluru-560068

**Divyashree. B. C**
Department of Biotechnology, The Oxford College of Engineering, Hosur road, Bommanahalli, Bengaluru-560068

**Amala Fathima**
Department of Biotechnology, The Oxford College of Engineering, Hosur road, Bommanahalli, Bengaluru-560068

**Priya J**
Department of Biotechnology, The Oxford College of Engineering, Hosur road, Bommanahalli, Bengaluru-560068

**ABSTRACT** *This work was aimed at isolating novel strains of Actinomycetes from soil samples of Western Ghats of Karnataka, capable of producing L-asparaginase. Soil samples used for the isolation were collected from of Shivamogga, Karnataka. 48 Actinomycetes strains were isolated by plating soil on Starch- Casein Agar media and screened for their ability to produce L-asparaginase using Asparagine dextrose salts agar. Enzyme activity was assayed by phenate method using ammonium chloride standard curve. Purification of the enzyme was carried-out by ammonium sulphate precipitation method, followed by dialysis. Specific activity of the enzyme was found to be 1.25 IU/ml. The highest producer among the isolates characterized upto genus level by Biochemical and slide culture method having Bergey's manual as reference. The isolate was found to belong to genus Streptomyces. Media optimization was done for submerged state fermentation and solid state fermentation.*

**INTRODUCTION:** The enzyme L-asparginase has attracted much attention in the past decades because of its antineoplastic activity (Sahu et al., 2007). . More attention has been given to isolate L-asparaginase from microorganism (Hill et al., 1967).Bacterial L-asparginase is the enzymes of high therapeutic value due to their use in treatment of certain kind of cancer therapies.The biochemical and kinetics vary with the microbial source. Bacterial L-asparaginase could cause an allergic reaction like skin rash, difficulty breathing, decreased blood pressure, unconsciousness, interference with blood clotting, increased blood sugar levels and liver enzymes and striking response to large dosage, with rapid necrosis of lymph node masses and clearing of leukemic infiltrates (Bhaskar et al., 2011). Glutaminase activity of the enzyme has been implicated in causing serious side effects (Roth et al., 2010). So, it is needed to discover new source of L-asparaginase that are serologically different and having lower Glutaminase activity. Many reports suggest that Actinomycetes are good source of L-asparaginase (Khamna et al., 2009 and Basha et al., 2009). Apart from medicinal use, the enzyme is also used in baking industry to bring down the formation of acrylamide in the process of baking (Bansal et al., 2010). With the development of its new functions, a great demand for L-asparaginase is expected in coming years. So, the present study aims at identifying L-asparaginase producing Actinomycetes population from Western Ghats.

**MATERIAL AND METHODS:**

**Isolation of actinomycetes:** Soil samples were collected aseptically at 15cm depth from surface, in sterile plastic containers from Western Ghat's regions of Shivamogga district, Karnataka and processed at the earliest (Augustine et al., 2004). Soil was subjected to serial dilution and lower dilutions were plated on Starch Casein Agar (SCA) Terbinafine(Singh et al., 2006). Plates were incubated at 27$^0$C for 15 days. The colonies having characteristics of Actinomycetes were isolated and pure cultures were maintained on Glycerol-Asparagine Agar (GAA) and stored at 4$^0$C.

**Screening for L-asparaginase activity:** Screening for L-asparaginase was carried-out; Isolates showing positive result were selected for L-asparaginase production by fermentation. The isolates were inoculated to ADS broth and incubated on orbital incubator shaker at 27$^0$C, at 200rpm for 10 days. After 10$^{th}$ day the biomass was separated by filtration and culture filtrate was assayed for Enzyme activity (Khamna et al., 2009).

**Assay of enzyme:** The available research papers on L-asparaginase assay had been following Nesslerization. But Nesslerization differ from other colorimetric estimation in that is peculiarly liable to give rise to turbid solution, so is not suitable for colorimetric estimation (Stanford, 1923). So we have followed the ammonia estimation by phenate method (Park et al., 2009). This method is usually carried out to estimate ammonia in drinking water. This reaction is based on the principle of Berthelot reaction that is the reaction of ammonia with phenol and hypochlorite to form blue indophenol in basic solution. Ammonia reacts with a hypochlorite to form a monochloramine which in turn reacts with two phenates to form an indophenol. Concentration of ammonia is determined by measuring the absorbance of indophenol at 630 nm.

The Standardized assay composition is as follows. 0.5 ml of culture filtrate was made up to 1ml with tris-Hcl buffer (pH-8.0) and mixed with 0.04M L-asparagine. This mixture was incubated for 10 minutes at 28$^0$C. After incubation reaction was stopped by adding 15% Trichloroacetic acid and centrifuged at 5000rpm for 10 minutes. 0.2 ml of the supernatant was made up to 1ml with tris-Hcl buffer (pH-8.0), 1ml sodium phenate, 0.1ml sodium nitroprusside, 0.5ml 4% sodium hypochlorite was added and incubated at 27$^0$C for 30 minutes. Intensity Blue colour developed was measured at 640nm. The enzyme activity was assayed using Ammonium chloride standard curve. One L-asparaginase unit (IU) is defined as that amount of enzyme which liberates 1µ mole of ammonia per minute under the optimal assay conditions.

**Media optimization:** Medium was optimized for both submerged fermentation (SMF) and solid state fermentation (SSF) by altering various parameters of the medium like Carbon source, nitrogen source, pH, temperature, inoculums

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size, trace salt solution and days of incubation. In SMF ADS broth is used as basal medium. In SSF corn cobs, Sooji, Soybean meal, black gingelly and groundnut meal were tried as substrates. 20g of sterilized substrate was mixed spore suspension. After incubation of 10 days the extracellular enzyme produced was extracted in 50ml 0.1M phosphate buffer (pH-7.5) (Basha et al., 2009) and assayed for enzyme activity.

**Partial Purification of enzyme:** The L-asparaginase enzyme precipitates out at 85-90% saturation (Basha et al., 2009) and protein precipitate was dissolved in 50mM Tri-HCl buffer (pH-8.5).It was dialyzed against same buffer till the complete exclusion of Ammonium sulphate. And enzyme activity was assayed. The total protein content of the partially purified enzyme was determined according to the Lowry et al., 1951. Specific activity of the enzyme was calculated.

Characterization of Organism: Characterization of the organism was done by according to Bergey's manual (2009) that is by growing the isolate on different nutrient media like Oat meal agar (OMA), GAA, Inorganic alts starch Agar (ISSA), slide culture method and performing various biochemical tests.

**RESULTS AND DISCUSSION:**

Total of 48 strains of Actinomycetes were isolated. Five isolates showed positive result for L-asparaginase production. Out of these five, two isolates namely AGB-11 and AGB-14 showed high enzyme activity (1.8 µg/ml/min and 2 µg/ml/min respectively) in ADS broth. And Media optimization was done for these two isolates and results presented in Table 1. The optimized medium for both the organisms was, l-asparagine 1%,yeast extract 1%, malt extract 1%, potassium nitrate 1%, trace salts solution 0.1%, pH-8.0, temperature 27$^0$C, incubation time 10 days and the enzyme activity obtained with this medium was 4.9 µg/ml/min, 5.5 µg/ml/min for AGB-11 and AGB-14 respectively. It can be inferred that the organism can better produce the enzyme in presence of organic and inorganic nitrogen sources than in presence of carbon source.

**Table 1: Results of media optimization for submerged fermentation**

| Variables | Substrate used | Enzyme activity (µg/ml/min) | |
|---|---|---|---|
| | | AGB-11 | AGB-14 |
| Carbon source | Starch 0.2% | 1.1 | 1.3 |
| | Starch 1% | 1.0 | 1.3 |
| | Glucose 0.2% | 1.8 | 2.0 |
| | Glucose 1% | 1.4 | 1.6 |
| Nitrogen source | Yeast extract 0.2% | 2.1 | 2.1 |
| | Yeast extract 1% | 2.2 | 2.5 |
| | Malt extract0.2% | 2.1 | 2.2 |
| | Malt extract 1% | 2.3 | 2.4 |
| | Potassium nitrate 0.5% | 2.3 | 2.3 |
| | Potassium nitrate 1% | 2.5 | 2.5 |
| | L-asparagine 1% | 1.8 | 2.0 |
| | L-asparagine 2% | 1.8 | 2.0 |
| Trace salt solution | 0.1% | 2.1 | 1.9 |
| | 0.5% | 1.1 | 0.9 |
| | 1% | 0.7 | 00 |
| pH | 6.0 | 00 | 1.1 |
| | 7.0 | 1.0 | 2.0 |
| | 8.0 | 1.7 | 2.0 |
| | 9.0 | 1.7 | 1.8 |
| Temperature In $^0$C | 25 | 1.1 | 2.2 |
| | 27 | 2.0 | 1.9 |
| | 30 | 1.9 | 0.8 |
| | 35 | 0.5 | 2.2 |
| Incubation time | 10$^{th}$ day | 2.0 | 2.2 |

Only AGB-14 was selected for SSF as it was the better producer in SMF. Out of 5 substrates tried Black till was selected as the substrate for media. Maximum production of L-asparaginase was found with a pH of 8.0 and 90% of moisture content in the medium.Change in the inoculum size did not show any significant effect on L-asparaginase production. 0.1ml of trace salt solution supplemented in the medium showed high enzyme activity. Addition of L- asparagine to substrate medium gave more yield of enzyme as it may act as inducer for the enzyme production. Optimium temperature for enzyme production was 27°C. 11 day is the optimum incubation period for AGB-14. Results of SSF are indicated in table 2. Optimized medium for SSF has following components, black till 20g, L-asparagine 1g, Trace salt solution 0.1ml, pH-8.0, temperature 27$^0$C, incubation time 11 days, the enzyme activity with this optimized media was 10.7 µg/ml/min.

**Table 2: Results of media optimization SSF**

| Variables | Concentration | Enzyme activity (µg/ml/min) |
|---|---|---|
| | | AGB-14 |
| Moisture content | 70% | 4.2 |
| | 80% | 4.9 |
| | 90% | 6.2 |
| | 100% | 5.0 |
| Inoculums size(X10$^4$) | 75 | 5.9 |
| | 100 | 6.0 |
| | 150 | 6.0 |
| | 200 | 6.1 |
| Trace salt solution | 0.1% | 5.8 |
| | 0.5% | 4.23 |
| | 1% | 3.0 |
| pH | 6.0 | 00 |
| | 7.0 | 1.2 |
| | 8.0 | 6.5 |
| | 9.0 | 6.0 |
| Temperature In $^0$C | 25 | 5.6 |
| | 27 | 6.4 |
| | 30 | 6.0 |
| | 35 | 5.1 |
| L-asparagine | 0.5g | 6.5 |
| | 1g | 7.2 |
| Incubation time | 11$^{th}$ day | 6.4 |

Enzyme activity of partially purified protein (PPE) was found to be 11.23µg/ml/min. The enzyme activity was found to increase after partial purification.The amount of protein in the PPE was found to be 9 µg. The specific activity of the enzyme by SSF was found to be 1.25 IU/ml.

Morphological characters of the isolates are presented in table 3. Slide culture of the organisms showed circular spores, arranged in the form of open spiral was observed, and the spore diameter was equal to diameter of hyphae. Both the isolates were negative for citrate utilization, positive for casein hydrolysis, starch hydrolysis, liquefaction of gelatin and lipase and lecithinase production. By comparing all results obtained with Bergey's manual the isolates were identified as Streptomyces spp.

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**Table 3: Morphological characters of the isolates on different medium**

| Isolate | SCA | OMA | GAA | ISSA |
|---|---|---|---|---|
| AGB-11 | Flat, elevation,entire margin, grey coloured, powdery spores, 1mm diameter | 5mm diameter, elevated, entire margin, dark grey sporulation, colourless, watery exudates on the colony, velvety, luxuriant growth. | Poor growth, pin head shape, no sporulation, penetrated into agar, entire margin, | Moderate growth, 5mm diameter, umbonate, white colour sporulation, entire margin. |
| AGB-14 | 1mm diameter, highly elevated grey coloured cottony sporulation entire margin | Luxuriant growth, 7mm diameter, highly elevated, sporulation initially white turning to grey. | Moderate growth, white colour sporulation, elevated, entire margin, 2mm diameter, penetrated into medium. | Luxuriant growth, powdery, 5mm diameter, elevated, irregular margin, colourless watery exudates on the colony. |

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