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Research Article

Isolation, production and partial purification of l-asparaginase from P*seudomonas aeruginosa* by solid state fermentation

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Abstract: L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an extracellular enzyme having potential therapeutic applications. In the present study, production and partial purification of L-asparaginase from*Pseudomonas aeruginosa*using solid state fermentation was carried out. It is inferred thatthe bacterial growth was observed in cetrimide agar medium after 48 hours of incubation. The isolate*Pseudomonas aeruginosa* was identified based on the morphological and biochemical characteristics. The isolated strainwas subjected to solid fermentation techniques along with Soya bean meal maker as substrate. The high amount of enzyme, L- asparaginase production was observed on optimal medium under 30 minutes incubation. Further, the enzyme activity was recorded maximum at 40ºC. Similarly the molecular weight of enzyme was confirmed following the enzyme pattern analysis using SDS PAGE and found to be 39000 Daltons.
Keywords: L-Asparaginase, Solid state fermentation, SDS-PAGE, *Pseudomonas aeruginosa*

INTRODUCTION

Enzymes are naturally occurring proteins produced by living organisms and function as biochemical catalyst.L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1), an extracellular enzyme having potential therapeutic applications, catalyses the breakdown of asparagine to aspartic acid and ammonia. Cancer cells cannotproduce L-asparagine, and mainly depends on theL-asparagine from the circulating plasma pools and in this way they are discriminated from normal cells in reduced expression of L –asparagine [1, 2]. L-Asparaginase from two bacterial sources (*E. coli* and *Erwiniacarotovora*) is in clinical use for the treatment ofacute lymphoblastic leukemia [3]. It is also used for the treatment of pancreatic carcinoma [4] and bovine lymphosarcoma [5].

L-Asparaginase is produced by a large number of microorganisms that include *E. coli* [6,7], *Erwinia cartovora* [8,9], *Enterobacter aerogenes* [10], *Corynebacterium glutamicum* [11], *Candida utilis* [12], *Staphylococcus aureus* [13], *Thermus thermophilus* [14] and *Pisum sativum* [15].These types of microbial enzymes have the enormous need, being able to be produced in large quantites by estabilished fermentation techniques [16].Solid-state fermentation is a very effective technique as the yield of the product is many times higherwhen compared to that in Submerged fermentation [17], and it also offers many other advantages [18].

The present study was to isolate *Pseudomonas aeruginosa* from soil sample and to produce asparaginase using various substrates and also optimise the physiological conditions for maximum enzyme activity (u/ml). Partial purification of the enzyme was done and the molecular weight determined by using SDS – PAGE.

MATERIALS AND METHODS

Sample collection and screening of microorganisms

Soil samples were collected in a sterilized polythene bag from the Hindusthan gardens and transported to the Biotechnology Department laboratory for further studies. The soil sample contains numerous microorganisms like bacteria, fungi and actinomycetes. 1gm of soil was weighed and transferred into a 250ml conical flask containing 100ml sterile distilled water and the dilution is 10^{-2}. One loop full of sample was taken from the above dilution, streaked onto cetrimide agar and incubated at 37^{o}C for 48 hrs.

Morphological and Biochemical Identification of *Pseudomonas aeruginosa*

Macroscopic observation was done by colony morphology such as size, shape, color etc. On the other hand, the cultural identification was made by streaking and observing the colonies under trans-illuminator. Biochemical characterization was carried out using standard procedures [19].

Asparaginase production by solid state fermentation

10gm Soya bean extract of 0.4-0.8cm particle size were moistened with 10ml of 0.01M of phosphate buffer (pH 7.4) and transferred to 250ml Erlenmeyer flask. The fermentation media was sterilized at 121°C

for 15 minutes.The flasks were inoculated with 3ml of prepared bacterial suspension and incubated at 37°C for four days. The extracellular crude enzyme was produced at the end of the fermentation period by adding 90ml of phosphate buffer (0.01M and pH 7) to the fermentation medium and shaking for 15 min followed by centrifugation at 8,000 rpm for 20 minutes. The cell free supernatant was used for crude enzyme preparation.

Purification of L-asparaginase By Ammonium sulfate fractionationmethod

The purification of L asparaginase from crude extract was made by ammonium sulfate fractionation method at 4°C. Fine powder of ammonium sulfate was added to the supernatant for saturation and the mixture was left for 24hr at 4°C followed by centrifugation at 8,000 rpm for 20 min at 4°C. The precipitate was dissolved in a 0.01M phosphate buffer (pH 8.5) and dialyzed overnight against the same buffer at 4°C.

Calculation of enzyme activity

The enzyme activity was calculated by using the following formula:

$$\text{Enzyme activity (u/ml)} = \frac{\text{Amount of } NH_4 \text{ liberated}}{\text{Incubation time} \times \text{ml of enzyme taken for test}}$$

Effect of glucose and corn steep liquor on asparaginase activity

To determine the effect of glucose and corn steep liquor on asparaginase production and activity, they were incorporated into production medium in separate flasks and the production medium without glucose and corn steep liquor used as control. After incubation conditions, the enzyme activity determined spectrophotometrically.

In the sample tube, 0.1ml of enzyme was taken along with 1.0 ml of Tris buffer solution and 0.1ml of asparagine with 0.90ml deionized water. The reaction mixture was incubated at 37°C for 30 minutes. The sample and control tubes were allowed to centrifuge for few minutes to clarify the enzyme.

Each tube (sample and control) containing 0.2 ml of supernatant was mixed with 4.30 ml of distilled water and then added 0.5ml of Nessler's reagent. The contents in the tube were mixed by inversion for 1 minute and the absorbance was noted at 436*nm*.

OPTIMISATION OF ASPARAGINASE ACTIVITY

Effect of time interval on Asparaginase Activity

The effect of time interval on asparaginase production was measured with two sets of tubes at different time intervals *viz*., 0, 10, 20, 30, 40, 50 and 60 minutes. The first tube (sample) containing 0.1ml of enzyme, 1ml of phosphate buffer, 0.1ml asparagine and 0.90 ml deionized water was incubated at 37°C at different time intervals. In the second tube (control) containing 0.1ml of enzyme without substrate was incubated at different time intervals [17].

Effect of temperature on asparaginase activity

To test the effect of temperature on asparaginase production, two set of tubes were prepared as above (sample and control). These tubes were kept at different temperatures like 20°C, 30°C, 40°C, 50°C, and 60°C. After the incubation, enzyme activity was measured.

Determination of Molecular Weight of Asparaginase Enzyme by SDS-PAGE

The Molecular pattern of asparaginase enzyme was analyzed using SDS-PAGE. The pre chilled enzyme extract was mixed with chilled phosphate buffer (pH 8.5) and deep freezed overnight. After incubation, 25µl of sample was loaded on the lanes (2-8) along with coloured protein marker in lane 1. Following the electrophoration, the gel was stained with Coomassie Brilliant Blue staining solution, destained and bands were observed [20].

RESULTS AND DISCUSSION

Isolation And Characterisation Of Bacteria From Soil

The bacterial growth was observed after 48 hours incubation period at 37°C in cetrimide agar medium. In macroscopic observation, the colonies appeared yellowish in colour or white to yellow colour. However, the flourescence colonies were observed by transilluminator analysis. The selected isolate was tested and compared with standard description. Based on these characters the isolated colonies were confirmed as *Pseudomonas aeruginosa*.

Effect of Various Physiological Conditions on Enzyme Activity

The asparaginase activity in the production medium without any supplement was 7units/ml. The enzyme activity was decreased (3.67units/ml) with the addition of glucose whereas the enzyme activity was enhanced, while the addition of corn steep liquor (10units/ml) (Table 1) (Figure 1).

Table 1: Asparaginase Activity [Control, Glucose, Corn Steep Liquor]

Substrate	Enzyme activity
Purified enzyme (Control)	7
Effect of glucose	3.67
Effect of Corn steep liquor	10

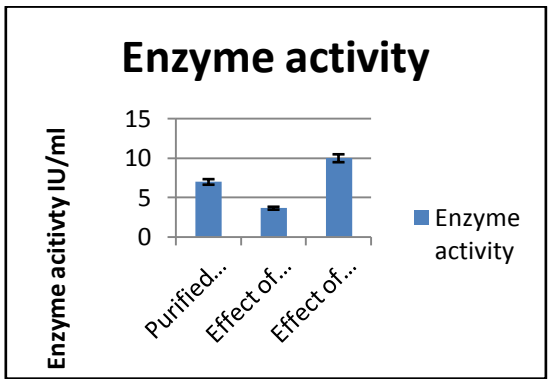

Figure 1: Effect of Glucose and Corn Steep Liquor On Asparaginase

To assertain optimum conditions for asparaginase production, their activity was tested at different physiological conditions. The most effective production was observed 30 minutes after incubation (Table 2). The maximum enzyme activity was observed at 30 minutes (0.90units/ml**)** whereas at 0 and 60 minutes the enzyme activity was found to be very low (0and 0.10 units/ml respectively).The asparaginase activity at 10 and 20 minutes was 0.20and 0.60 units/ml respectively. Similarly, at 40 and 50 minutes the asparaginase activity was found to be 0.50 and 0.20 units/ml respectively (Figure 2).

Table 2: Asparaginase Activity of *Pseudomonas Aeruginosa* at Different Time Intervals

Time in minutes	0	10	20	30	40	50	60
Asparaginase activity	0	0.22	0.62	0.94	0.51	0.20	0.18

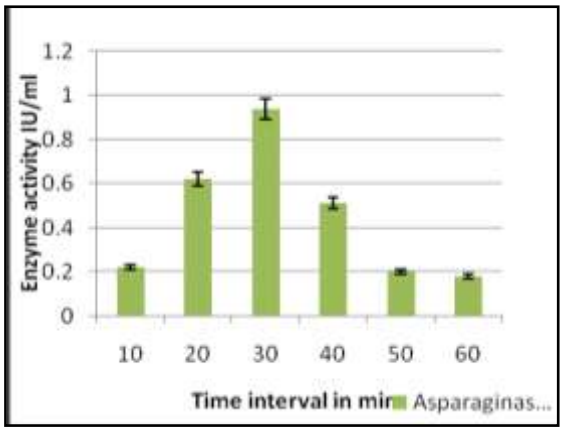

Figure 2: Asparaginase Activity at Different Time Intervals

The most conductive production of enzyme asparaginase was observed at 40ºC (Table 3). The maximum enzyme activity was (0.57units/ml) whereas the asparaginase activity at 20°C and 30°C was found to be very low (0.11 and 0.42 units/ ml respectively). However, the activity of asparaginase at 50°C and 60°C was appreciable (0.53and 0.21 units/ml) respectively. The maximum enzyme activity for asparaginases at 37ºC was observed in *Pseudomonas stutzeri*MB-405 [21] and *Staphylococcus* [22] (Figure 3).

Table 3: Asparaginase Activity of *Pseudomonas Aeruginosa* at Different temperature

Temperature	20	30	40	50	60
Asparaginase Activity	0.11	0.42	0.57	0.53	0.21

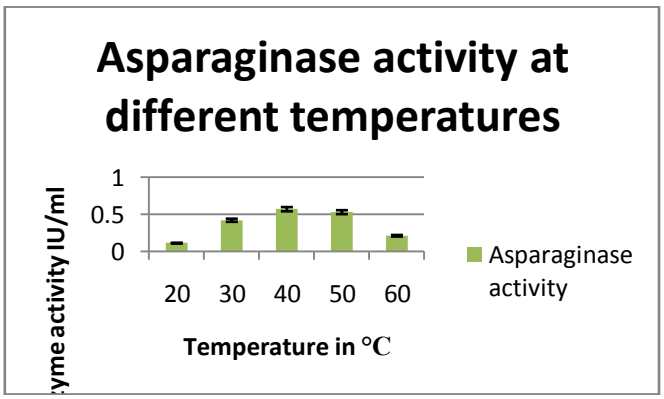

Figure 3: Effect of Temperature on Enzyme Activity

Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. This system offer numerous advantage like high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement of simple fermentation equipments etc.With the

development of its new functions, a great demand for L-asparaginase is expected in the coming years.

The biochemical and enzyme kinetic properties vary with the microbial source. However, *Erwinia* asparaginase had a shorter half- life than *E. coli* .The pH 9 was found to be most effective for the production of asparaginase. On the other hand, the enzymes activity was appreciable in pH 5,6 and 8. Similarly the higher enzyme activity was also observed on the same pH. The results are in accordance with that obtained with *E. coli* [23] and [24] *Pseudomonas aeruginosa* 10145 [25] and other microbial asparaginase activities [26]. SDS – PAGE reveals the molecular weight of enzyme to be 140 kilodaltons when compared with the protein marker (Figure 4)

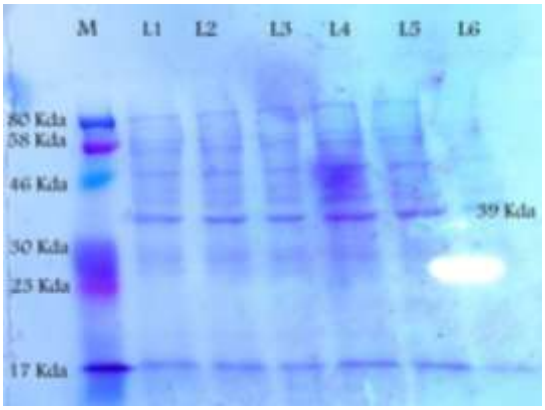

Figure 4: Molecular Profiling on SDS – Page

CONCLUSION

The present investigation reports that soil isolate, *Pseudomonas aeruginosa* produced asparaginase in 30 minutes incubation with maximum activity at 40^{O}C. The enzyme was precipitated using ammonium sulphate and molecular weight pattern observed on SDS – PAGE which indicates a single band of 39kDa. Further, analysis of the enzyme for its antitumour properties can be carried out.

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