

Screening and Optimization of L-Asparaginase Production From Bacillus Species

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Abstract: L-asparaginase is an anti-neoplastic agent used in lymphoblastic leukaemia chemotherapy. In the present work a total of 5 soil samples were collected from various places near to Aurangabad. Soil microbial isolates were screened for potential producers of L-asparaginase using a phenol red indicator growth medium and the microbe producing the largest hydrolysis zone was selected. The isolate was characterized by biochemical tests and was found to belong to *Bacillus* sp. From these soil samples 12 isolates were identified as *Bacillus licheniformis* (5), *Bacillus alvei* (2), *Bacillus megaterium* (1), *Bacillus cereus* (1), *Bacillus circulans* (1) and *Bacillus subtilis* (2). The optimization study for the process parameters was given useful information about the production of the enzyme maximally. Potential L-asparaginase producer isolates were showed the maximum activity at pH 7 and temperature at 37°C.

Keywords: L-asparaginase, anti-neoplastic agent, *Bacillus* species

I. Introduction

The enzyme L-asparaginase 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-asparaginase 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.

L-asparaginase is a relatively wide spread enzyme, found in many microorganisms such as *Aerobacter*, *Bacillus*, *Pseudomonas*, *Serratia*, *Xanthomonas*, *Photobacterium* (Peterson and Ciegler, 1969), *Streptomyces* (Dejong, 1972), L-Asparaginase has received increased awareness in current years for its anti carcinogenic potential. Cancer cells distinguish themselves from normal cells in diminished expression of L-asparagine (Swain *et al.* 1993; Manna *et al.*, 1995). Now a day's the use of L-asparaginase increases for various purposes and the demand of research work is to find out the potential L-asparaginase producers for commercial use. Hence the purpose of the present investigation is to isolate the bacillus species from soil and to enhance the production of L-asparaginase. This was the objectives of the present work.

II. Materials And Methods

Collection of Soil Samples

For the isolation of the L-asparaginase producing bacteria a total of five samples were collected in sterile plastic bags from different location of Aurangabad, India. The soil samples were immediately carried to the laboratory for further studies.

Isolation of microorganism and maintenance of culture

A known quantity (1g) of soil sample was serially diluted with sterile distilled water and grown on agar-based modified M9 medium (Prakasham *et al.*, 2010). The inoculated agar plates were incubated at 37°C overnight. A microbial culture that displayed pink red coloured colony was selected for further studies. The pinkish red colony (asparaginase-producing bacterial colony) picked from the plates and was streaked on nutrient agar slant. The isolates showing pink red colored colonies were grown at 37°C and after growth were stored at 4°C. The isolates were sub-cultured on fresh nutrient agar slants every fortnight. After isolation as a pure culture, the cultures were characterized using morphological and biochemical tests.

Production of L-asparaginase

L-asparaginase production by the isolate was carried out by submerged fermentation. The sterilised production media (composition (g/l): KH₂PO₄ 2.0, L-asparagine 6.0, MgSO₄.7H₂O 1.0, CaCl₂.2H₂O 1.0 and glucose/maltose 3.0) was inoculated with a loop-full of log phase bacterial culture and was incubated in a rotary shaker at 37°C at 200 rpm for 24 h.

Purification of L-asparaginase

The enzyme was purified by the following steps at 4°C. The fermentation broth was centrifuged at 10,000 g for 10 min. The purification was carried out using crude enzyme extract (Distasio et al., 1982). Finely powdered ammonium sulphate was added to the crude extract. The L-asparaginase activity was associated with the fraction precipitated at 70 - 100% saturation. The precipitate was collected by centrifugation at 9,000 g for 15 min, dissolved in sodium borate buffer and dialysed against the same buffer. The dialysed fraction was applied to a DEAE column, an anion exchanger, pre-equilibrated with Tris-HCl buffer, pH 8.6. The enzyme was eluted (1 ml/min) with NaCl gradient (0.1 - 0.5 M) and 0.1 M borate buffer, pH 7.0. The active fractions were collected, dialysed and concentrated.

Estimation of L-asparaginase activity

L-asparaginase enzyme assay was performed by a colorimetric method according to Wriston and Yellin (1973) at 37°C using an UV-Visible spectrophotometer by estimating the ammonia produced during L-asparaginase catalysis using Nessler's reagent. A reaction mixture consisting of 0.01 M L-asparagine and 0.05 M Tris-HCl buffer (pH 8.6) was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 15% trichloroacetic acid solution. The liberated ammonia was coupled with Nessler's reagent and was quantitatively determined using an ammonium sulphate reference standard. 1 unit of the L-asparaginase (IU) is defined as the amount of enzyme capable

Estimation of protein

The amount of protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Qualitative Determination of L-asparaginase activity

Modified M-9 agar medium was used for qualitative of L-Asparaginase. Stock solution (2.5%) of phenol red dye was prepared in ethanol and pH was adjusted to 7.0. From this, 0.3ml of dye (stock solution) was added to 100 ml of modified M-9 medium. Screened L-Asparaginase producer bacterial isolate was placed on the Modified M-9 agar medium and NaNO₃ (nitrogen source) was added as control to M-9 medium instead of L-Asparagines. All plates were incubated at 37°C for an 18h. Formation of pink zone around the bacterial colonies indicate the L-Asparaginase production by bacteria because at alkaline pH (due to accumulation of ammonia in medium) phenol red indicator was converted to pink.

Quantitative assay of L-asparaginase

The quantitative estimation of enzyme activity was done with selected culture Asp1, Asp3, Asp4, Asp7, Asp11. Asparaginase activity was measured by method of Mashburn and Wriston (1963). The rate of hydrolysis of L-asparagines was determined by measuring the release of ammonia using Nessler's reaction. The reaction mixture contained 0.5 ml of enzyme sample, 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.6) and 0.5 ml of 0.04 M L-asparagines. The reaction mixture was incubated at 37°C for 30 min. The enzyme activity was stopped by the addition of trichloroacetic acid (TCA 10% w/v). The mixture was then centrifuged at 10,000 rpm for 5 min, and 0.1 ml of the supernatant was taken and to it 3.7 ml of distilled water was added; 0.2 ml of Nessler's reagent was added to the reaction tube and kept at 20°C for 20 min. The absorbance was measured at 450 nm using spectrophotometer. The amount of ammonia liberated was calculated using ammonium standard curve. One unit of L-asparaginase activity is defined as release of one micromole of ammonia per hour at 37°C and pH 8.6.

Identification of bacterial strain

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.

Enzyme Assay

L-asparaginase activity was measured in terms of the rate of hydrolysis of L-asparagines by measuring the amount of ammonia released in the reaction, by the method of (Wriston 1970) utilizing Nesslerization, the most commonly used L-asparaginase assay. The reaction mixture consisted of 0.2 ml of 0.05 M Tris-HCl (pH 8.6), 1.7 ml of 0.01 M L-asparagines and 20µl of appropriately diluted enzyme. After incubation for 10 minutes at 37°C, the reaction was stopped by the addition of 0.1 ml of 1.5 M TCA (Trichloroacetic Acid). The contents were clarified by centrifugation and to 2.5 ml of the clear supernatant an equal volume of de-ionized water was added. To this mix, 0.5 ml of Nessler's Reagent was added and it was incubated at 37 °C for 10 minutes. The absorbance was taken at 480nm. One unit of enzyme activity is defined as the amount of enzyme that catalyses the release of 1µmol of ammonia at 37 °C.

III. Results And Discussion

In the present study, a total of 5 soil samples were collected in sterile plastic bags from different villages namely Shendra, Kumbhefal, Chikalthana, Warud, and Zalta near Aurnagabad city (MS) India and transported immediately to Laboratory for further study. These containers were maintained at 4°C or less to ensure the minimal biological activity. Processing of the samples for the isolation of bacteria was carried out within 3 hr of sample collection.

Physico-chemical Analysis of soil samples

Table 1: Physico-chemical characteristics of soil samples

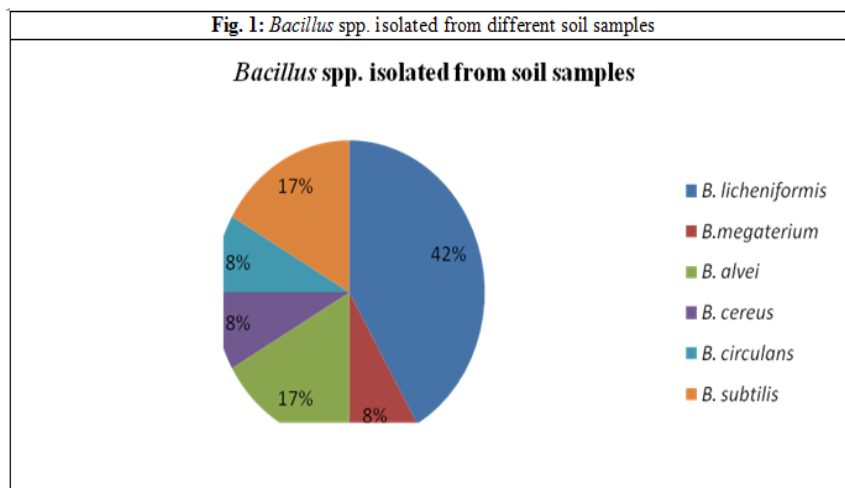
Sr. No.	Physico-chemical parameter	Soil Samples				
		Sample I	Sample II	Sample III	Sample IV	Sample V
1	Color	Black	Grayish	Brown	Black	Grayish
2	pH	6.7	8.0	9.0	6.9	7.2
3	Odour	Earthy	Earthy	Earthy	Earthy	Earthy
4	Temperature	25 ⁰ C	23 ⁰ C	30 ⁰ C	35 ⁰ C	30 ⁰ C

The physico-chemical characteristics of all five soil samples collected from different villages were showed that the samples were black, grayish, brown in color having pH varied from 6.7 to 9.0 and having earthy odor respectively. The temperatures of samples were also varied from 23⁰C to 35⁰C (Table 1).

Table 2: Isolation of L-Asparaginase producer Bacillus spp. from soil collected from various villages nearby Aurangabad

Sr. No.	Code of isolates obtained	Pink color formation on M9 Media	Probable Identification
1	Asp1	Positive	<i>Bacillus licheniformis</i>
	Asp2	Negative	<i>Bacillus megaterium</i>
	Asp3	Positive	<i>Bacillus alvei</i>
2	Asp4	Positive	<i>Bacillus licheniformis</i>
	Asp5	Negative	<i>Bacillus cereus</i>
3	Asp6	Negative	<i>Bacillus licheniformis</i>
	Asp7	Positive	<i>Bacillus circulans</i>
4	Asp8	Negative	<i>Bacillus licheniformis</i>
	Asp9	Negative	<i>Bacillus subtilis</i>
5	Asp10	Negative	<i>Bacillus licheniformis</i>
	Asp11	Positive	<i>Bacillus subtilis</i>
	Asp12	Negative	<i>Bacillus alvei</i>

From the total 5 soil samples collected from different villages near to Aurangabad, total 12 isolates were isolated in that five isolates namely Asp1, Asp3, Asp4, Asp7, Asp11 were found to be potential L-Asparaginase producers. All the remaining isolates (Asp2, Asp5, Asp6, Asp7, Asp8, Asp9 and Asp10) were unable to form the pink coloration after incubation on the modified M9 medium and considered as L-asparaginase negative (Table 2).

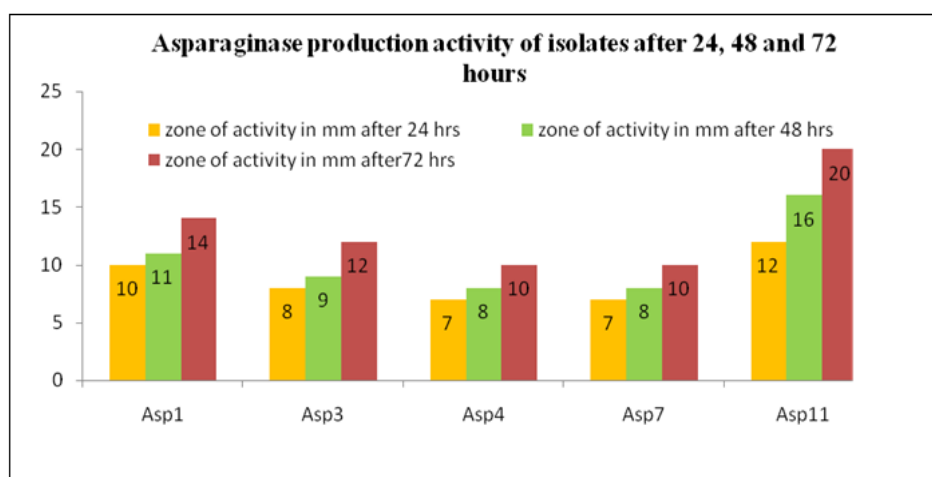


In the present work, from the five soil samples 12 *Bacillus* spp. were isolated, among them *B. licheniformis* (5, 42%), *B. alvei* (2, 17%), *B. subtilis* (2, 17%), *B. cereus* (1, 8%), *B. circulans* (1, 8%), *B. megaterium* (1, 8%) shown in (fig. 1).

Table 3: L-Asparaginase production activity of isolates after 24, 48 and 72 h. respectively

Sr. No.	Code of isolates	L-asparaginase production after 24 hour incubation (Zone of activity in mm)	L-asparaginase production after 48 hour incubation (Zone of activity in mm)	L-asparaginase production after 72 hour incubation (Zone of activity in mm)
1	Asp1	10	11	14
2	Asp3	8	9	12
3	Asp4	7	8	10
4	Asp7	7	8	10
5	Asp11	12	16	20

From the above (Table 3) it was observed that the isolates Asp1, Asp3 Asp4, Asp7, Asp11 showed 10, 8, 7, 7 and 12 mm of zone of activity respectively after 24 hours of incubation and after 48h zone of activity was 11, 9, 8, 8 and 16mm respectively whereas 14, 12, 10, 10, 20mm zone of activity respectively on the plate containing the M9 modified medium after 72h of incubation. This result shows that as the incubation time increases the activity of L-asparaginase production of the enzyme increases (fig. 5).



A total of 5 soil samples were collected from various places near to Aurangabad shown in fig. 1. From these 5 soil samples, a total of 12 isolates of *Bacillus* genera were obtained and identified on basis of standard morphological, biochemical and sugar fermentation characteristics by using determinative bacteriology of Bergey's manual (Table 5). All the isolates belong to the *Bacillus* genera as the isolation step involves the heat

treatment which excluded the other organisms. It was recorded that the isolates were *Bacillus licheniformis* (5), *Bacillus alvei* (2), *Bacillus megaterium* (1), *Bacillus cereus* (1), *Bacillus circulans* (1) and *Bacillus subtilis* (2). Table 3 shows the data regarding the screening of the isolates for the production of the L-Asparaginase enzyme it was recorded that the isolates Asp1, Asp3, Asp4, Asp7, Asp11 were showing 14, 12, 10, 10, 20 mm zone of activity respectively on the plate containing the M9 modified medium after 72 hours of incubation. . The Asp1, Asp4, Asp7, Asp11 was showing 10, 8, 7, 7, and 12 mm of zone of activity after 24 hours of incubation. This result shows that as the incubation time increases the activity of production of the enzyme increases. The optimization of the isolates to produce the one activity on different sugars has shown that the lactose was the only sugar showing the maximum zone of activity for all the isolates. Followed by the lactose the glucose has shown the fair zone activity. The media containing sugars dextrose and sucrose has shown the minimum production of L-Asparaginase.

Effect of the different pH on the activity of the isolates to produce the L-Asparaginase shown that the isolates were showing the maximum activity to pH 7 and activity was getting decreased as the pH either increase towards 9 or decreases towards 5. It was also observed that the isolate Asp11 was found to be the most efficient L-Asparaginase producer as it was showing the larger zones of activity at all pH compared to all other isolates. The effect of different temperatures on the L-Asparaginase producing activity of isolates was studied in detail by incubating the isolates at different temperatures. Table 8 represents the effects of different temperatures on the activity of the isolates to produce the L-Asparaginase and it was recorded that the maximum activity was found at temperature 37°C and the activity was decreasing as the temperature was either decreasing below 37°C or increasing above 37°C. The purification has been carried out by using series of methods to obtain the purified enzyme. The activity was reduced to 2211 U in the enzyme sample after CM-Sephadex C50 in comparison with the α - amylase activity of 4530 U of crude extract and the total protein content reduced to 26 mg in comparison with the initial protein conc. of 355.80 mg. However the specific activity at the end of process was found to be increased 85.038 U/mg compared to initial 12.73 U/mg at the crude extract. It was also indicated that the- amylase activity at the end of process was purified to 5.34 fold using CM-Sephadex C50 chromatography.

IV. Conclusion

It can be concluded that screening of the isolates for the production of L-Asparaginase at different incubation time was important in determining the efficiency of the isolates to produce the enzyme. The studies on the optimization was carried out and have shown that the enzyme can be produced at higher levels at pH around 7, temperature near about 37°C and using the sugar lactose. Hence the study concluded that Isolate Asp11 (*Bacillus subtilis*) have shown high degree production of the L-Asparaginase enzyme after the strain improvement by UV radiation and could be explored as suitable candidate bacteria for the commercial production of this medically important enzyme as it has been used in the treatment of the Leukemia as it possessing the antitumor activities.

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