WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 7, Issue 03, 1713-1722. Research Article ISSN 2277–7105

L-ASPARAGINASE: AN ULTIMATE ANTI-NEOPLASTIC ENZYME ISOLATED FROM *BACILLUS* SPECIES

S. A. Bhutada* and S. B. Dahikar

Department of Microbiology, Sanjivani Arts, Commerce and Science College, Kopargaon (INDIA).

Article Received on
20 Dec. 2017,

Revised on 10 Jan. 2018,
Accepted on 31 Jan. 2018

DOI: 10.20959/wjpr20183-11047

***Corresponding Author**
Dr. S. A. Bhutada
Department of Microbiology, Sanjivani Arts, Commerce and Science College, Kopargaon (INDIA).

ABSTRACT

L- asparaginase is enzyme acting on L- asparagine and is widely used as anticancer agent. The reason it is preferred for the purpose is it is biodegradable, non-toxic and can be administered at the local site quite easily. At present various microorganisms are used for production of L-asparginase. The main objectives of this study were to determine anti-neoplastic activity of enzyme - L-asparaginase from Bacillus species from soil.In the present study, a total of five soil samples were collected from various places near to Kopargaon, 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-asparginase producer isolates were showed the maximum activity at pH 7 and temperature at 37°C. After that the isolates were exposed to the UV treatment for 5, 10 and 15 min for strain improvement and among them Asp11 *(Bacillus subtilis)* was found useful strain for the commercial production of the L-Asparaginase. The purified enzyme of Asp11 was used for protein profile. This highly potential species can be used for the commercial production of the enzyme which is having immense medical applications as it is one of the promising treatments for the threatening disease called as cancer.

KEYWORDS: L-asparginase enzyme, Anti-neoplastic acivity, *Bacillus* species.

INTRODUCTION

Microbes are the potential source for industrial and clinically important enzymes. The inability of the animal and plant sources to meet the demand of enzymes has lead to the increased interest in microbial sources. Microbial enzymes are preferred over plant or animal enzymes due to their economic production, consistency, high stability, ease of process modification and optimization. Microbial enzymes provide a greater diversity of catalytic activities and wide range of specificities to perform their biochemical reactions. The enzyme L-asparginase has attracted much attention in the past decades because of its antineoplastic activity (Sahu *et al.*, 2007).

L-Asparaginase is the first enzyme with anti-leukemic activity to be intensively studied in human beings (Savitri *et al.*, 2003). Since extraction of L-asparaginase from mammalian cells is difficult, microorganisms have proved to be a better alternative for L-asparaginase extraction, thus facilitating its large scale production. Bacterial L-asparginase is the enzymes of high therapeutic value due to their use in treatment of certain kind of cancer therapies. 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). Most of the therapeutic and medicinally important enzymes are produced by microorganisms which have been commonly used for anti-inflammatory, thrombolytics or anticoagulants and oncolytics as replacement for metabolic deficiency. A major application of therapeutic enzymes is in the treatment of cancer. L-asparaginase has been widely used in chemotherapy in the field of medicine which has been proved to be a potential enzyme for the treatment of acute lymphocytic leukaemia (ALL) and Lymphosarcoma cancer. Microbial sources are most common for large scale production of L-asparaginase, because of the cost effective production. Now a day's the use of L-asparginase increases for various purposes and the demand of research work is to find out the poterntial L-asparginase 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.

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 Kopargaon, 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 37^0C 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 37^0C and after growth were stored at 4^0C. 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 thefraction 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-Asparginase. 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-Asparginase producer bacterial isolate was placed on the Modified M-9 agar medium and $NaNO_3$ (nitrogen source) was added as control to M-9 medium instead of L-Asparagines. All plates were incubated at 37^0C 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 37^0C for 30 min. The enzyme activity was stopped by the addition of trichloroacetic acid (TCA 10%w/v). The mixture was then centrifugated 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 37^0C 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 37^0C, 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 0C 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 ^{0}C.

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 4^0C 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	25^0C	23^0C	30^0C	35^0C	30^0C

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 23^0C to 35^0C (Table 1).

Table 2: Isolation of L-Asparginase 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	*Bacillus licheniformis*
	Asp2	Negative	*Bacillus megaterium*
	Asp3	Positive	*Bacillus alvei*
2	Asp4	Positive	*Bacillus licheniformis*
	Asp5	Negative	*Bacillus cereus*
3	Asp6	Negative	*Bacillus licheniformis*
	Asp7	Positive	*Bacillus circulans*
4	Asp8	Negative	*Bacillus licheniformis*
	Asp9	Negative	*Bacillus subtilis*
5	Asp10	Negative	*Bacillus licheniformis*
	Asp11	Positive	*Bacillus subtilis*
	Asp12	Negative	*Bacillus alvei*

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-Asparginase 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).

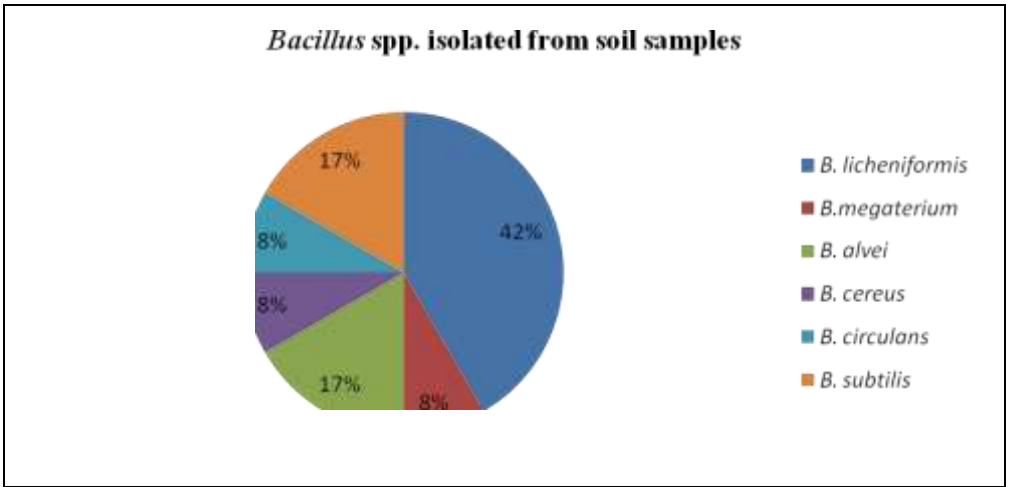

Fig. 1: *Bacillus* spp. isolated from different soil samples

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-Asparginase production activity of isolates after 24, 48 and 72 h. respectively

Sr. No.	Code of isolates	L-asparginase production after 24 hour incubation (Zone of activity in mm)	L-asparginase production after 48 hour incubation (Zone of activity in mm)	L-asparginase 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 repectively 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-asparginase production of the enzyme increases (Fig. 5).

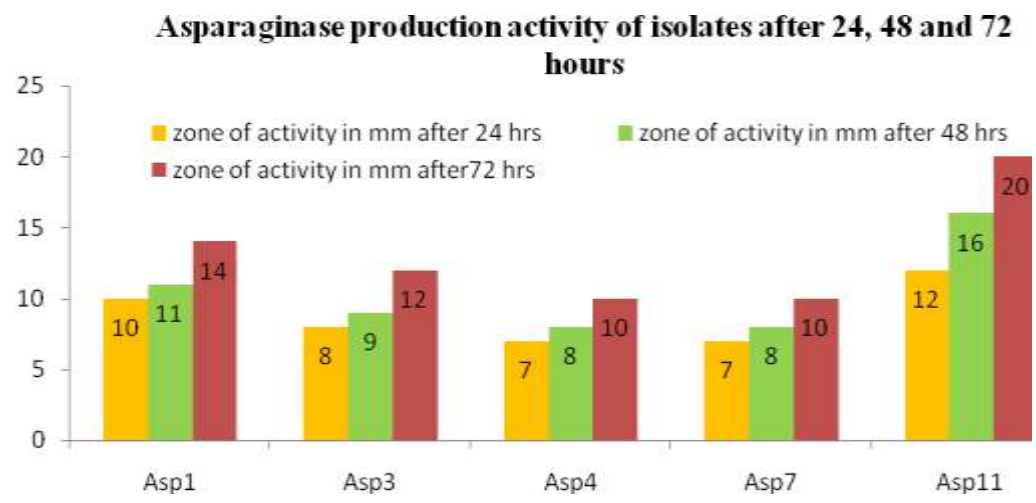

Fig. 5: L-Asparaginase production activity of isolates after 24, 48 and 72 hours respectively.

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-Asparginase 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-Asparginase.

Effect of the different pH on the activity of the isolates to produce the L-Asparginase 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-Asparginase 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-Asparginase 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-Asparginase 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.

CONCLUSION

It can be concluded that screening of the isolates for the production of L-Asparginase 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 37^0C and using the sugar lactose. Hence the study concluded that Isolate Asp11 (*Bacillus subtilis*) have shown high degree production of the L-Asparginase 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.

REFERENCES

1. Augustine, S, K., Bhavsar,S,P., Baserisalehi,M.,and Kapadnis,B.P.(2004). Isolation, characterization and optimization of an actinomycete of soil origin. Indian J Exp Biol, 42: 928-32.
2. Bansal,S., Gnaneswari,D., Mishra,P. and Kundu,B. (2010). Structural stability and functional analysis of L-Asparaginase from Pyrococcus furiosus. Biochemistry (Mosc), 75(3): 375-381

3. Basha,N,S., Rekha,R., Komala,M. and Ruby, S.(2009). Production of extracellular anti-leukaemic enzyme L-asparginase from marine actinomycetes by solid-state and submerged fermentation. Tropical journal of Pharmaceutical Research, 8(4): 353-360.
4. Baskar, G., and Renganathan,S. (2011) Optimization of Media Components and Operating Conditions for Exogenous Production of Fungal L-asparaginase Chiang Mai J. Sci, 38(2): 270-279.
5. Bergey's manual ofdeterminative bacteriology, (2000). Actinomycetales. 9th edition.
6. Hill,J., Roberts, J., Loeb, E., Kahn, A. & Hill,R. (1967). L-asparaginase therapy for leukemia and other malignant neoplasm, JAMA, 202: 882.
7. Khamna, S.,Yokota, A., & Lumyong, S.(2009). L-asparginase production by actinomycetes isolated from some Thai medicinal plant rhizoshere soils. IJIB, 6-1, 22.
8. Lowry, O, H., Rosebrough, N, J., Farr,A,L.and Randall,R,J. (1951). Protein measurement with the folin-phenol reagent J. Biol. Chem, 48: 17-25.
9. Park,G., Oh,H. and Ahn,S. (2009). Improvement of the Ammonia Analysis by the Phenate Method in Water and Wastewater. Bull. Korean Chem. Soc., 30(9): 12. 12.
10. Roth, G., Volpato, G., Neves, C, E., Renard, G., Basso, L, A., Chies, J,M., and Santos, D,S. (2010). Expression patterns of recombinant asparaginase in different E.coli strains. PUCRS.
11. Sahu, M,K., Sivakumar, K., Poorani, E., Thangaradjou, T., & Kannan, L. (2007). Studies on L-asparaginase enzyme of actinomycetes isolated from esturine fishes, Journal of Environmental Biology, 28(2): 465-474.
12. Savitri A. N, Azmi W (2003). Microbial L-asparaginase: A potent antitumor enzyme. Indian J. Biotechnol, 2: 184-194.
13. Singh,S,L., Baruha,I. and Bora,T,C. (2006). Actinomycetes of loktak habitat isolation and screening of antimicrobial activity. Biotech, 5(2): 217-221.
14. Stanford, V, R. (1923). Nesslerisation and avoidance of turbidity in nesslerised solutions. Biochem J, 18(2): 460-461.