ISSN: 0976-3031

Available Online at http://www.recentscientific.com

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 8, Issue, 6, pp. 17698-17702, June, 2017

International Journal of Recent Scientific Research

DOI: 10.24327/IJRSR

Research Article

IMPROVEMENT OF *BACILLUS* SPECIES BY MUTATION FOR PRODUCTION OF L-ASPARGINASE

Patil R.C[1,2] and Jadhav B. L[2]

[1]Department of Microbiology, Bhavan's College, Andheri (West), Mumbai, Maharashtra-400058, India
[2]Department of Life Sciences, University of Mumbai, Kalina, Mumbai, Maharashtra 400098, India

DOI: http://dx.doi.org/10.24327/ijrsr.2017.0806.0407

ARTICLE INFO

Article History:

Received 05th March, 2017
Received in revised form 08th April, 2017
Accepted 10th May, 2017
Published online 28st June, 2017

Key Words:

Strain Improvement, *Bacillus* species, mutation, L-asparginase enzyme

ABSTRACT

L-Asparaginase has received increased awareness in current years for its anti carcinogenic potential. In the present work a total of 5 soil samples were collected from various places near to Aurangabad, 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. 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.

INTRODUCTION

L-Asparaginase has received increased awareness in current years for its anti carcinogenic potential. Since several types of tumour cells require L-asparagine for protein synthesis, they are deprived of an essential growth factor in the presence of L-asparaginase, thus, resulting in cytotoxicity of leukaemic cells. L-asparaginase is a relatively wide spread enzyme, found in many microorganisms such as *Aerobacter, Bacillus, Pseudomonas, Serratia, Xanthomonas, Photobacterium* (Peterson & Ciegler, 1969), *Streptomyces* (Dejong, 1972), *Proteus* (Tosa *et al*, 1971), *Vibrio* (Kafkewitz & Goodman, 1974) and *Aspergillus* (Sarquis *et al*, 2004). The fact that not all L-asparaginase possess antitumour properties seems to be related to the affinity of the enzyme for the substrate and factors affecting the clearance rate from the system (Cornea *et al*, 2002). Recently, it has been shown that L-asparaginases derived from *E. coli* and *Erwinia carotovora* possess antitumor activity particularly against acute lymphoblastic leukemia (Mashburn and Wriston, 1964).

Strain improvement can generally be described as the use of any scientific techniques that allow the isolation of cultures exhibiting a desired phenotype. There are various isolates usually producing commercially important products in very low concentrations and therefore every attempt is made to increase the productivity of the chosen organism. Increased yields may be achieved by optimizing the culture medium and growth conditions, but this approach will be limited by the organism's maximum ability to synthesize the product. The potential productivity of the organism is controlled by its genome and, therefore, the genome must be modified to increase the potential yield (Moorthy *et al,* 2010). Now a day's the use of L-asparginase increases for various purposes and the demand of research work is to find out the potential L-asparginase producers for commercial use. In this study we tried to improve L-asparginase production from *Bacillus* spp. by using different physical and chemical mutagen.

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 37^0C overnight. A microbial culture that displayed pink red colored colony was selected for further

Corresponding author:* **Patil R.C
Department of Microbiology, Bhavan's College, Andheri (West), Mumbai, Maharashtra-400058, India

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.

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.

Strain improvement

Strain improvement refers to use of any specific technique that allows the isolation of cultures exhibiting the desired phenotype.

The natural isolate obtained after primary and secondary screening usually produce commercially important products in very low concentration and therefore every attempt is made to increase the productivity of organism. Increased yield may be achieved by optimizing the culture medium and growth condition but the approach is limited by microorganism to synthesize the product called as productivity.

CM-Sephadex C50 ion-exchange chromatography

The concentrated enzyme solution was applied to the column of CM-Sephadex C50 that was pre-equilibrated with a 0.01 M phosphate buffer, pH 8.5. It was eluted with the NaCl gradient (0.1-0.5 M) and 0.1M borate buffer, pH 7. The active fractions were collected, dialyzed, concentrated, and lyophilized. This preparation was used in the subsequent step. The enzyme was assayed by the direct nesslerization method according to the method of Sinha *et al*., (1991).

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 locations of Aurangabad city (MS) India and transported immediately to the laboratory. 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

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). 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.

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

Table 2 Isolation of L-Asparginase producer *Bacillus* spp. from soil collected from various villages nearby Aurangabad City

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
	Asp10	Negative	Bacillus licheniformis
5	Asp11	Positive	Bacillus subtilis
	Asp12	Negative	Bacillus alvei

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).

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 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-asparginase production of the enzyme increases.

Strain improvement by UV Radiation

To increase the production the wild organism was subjected to UV mutation. UV rays are important inducers of strain mutations. The pyrimidines (thymine and cytosine) are especially sensitive to modifications by UV rays absorption. This may result in the production of thymine dimers that distort the DNA helix and block future replications. In many cases, mutations by UV are harmful, but occasionally it may lead to a better adapted organism to its environment with improved bio catalytic performance. The potential of a microorganism to mutate is an important property conferred by DNA, since it creates new variations in the gene pool (Parekh *et al.,* 2000).

The activity of the isolates to produce L-asparaginase incubated for 24 hrs. after UV treatment for 5, 10 and 15 min was recorded in table 5 it was recorded that Asp1, Asp3, Asp4 were unable to produce the enzyme after 15 min of UV exposure. Isolates Asp7, and Asp11 were showing the enzyme producing activity even after the UV treatment of 15 min and showing the zone of activities as 10 and 16 mm respectively (Table 4).

Table 4 L-asparaginase production activity of isolates after 24 hours followed by exposure of UV after 5, 10 and 15 min. respectively

S N	Code of isolates	L-Asparginase production after 5 min UV exposure	L-Asparginase production after 10 min UV exposure	L-Asparginase production after 15 min UV exposure
1	Asp1	10	12	0
2	Asp3	8	9	0
3	Asp4	7	8	0
4	Asp7	7	8	10
5	Asp11	12	14	16

Table 5 L-asparginase production activity of isolates after 48 hours followed by exposure of UV after 5, 10 and 15 min respectively

Sr. No.	Code of isolates	L-Asparginase production after 5 min UV exposure	L-Asparginase production after 10 min UV exposure	L-Asparginase production after 15 min UV exposure
1	Asp1	10	14	0
2	Asp3	8	11	0
3	Asp4	7	10	0
4	Asp7	7	11	10
5	Asp11	12	16	20

The results UV exposure after 48 hours of incubation was recorded in (Table 5). It showed the increasing activity compared to the 24h incubation. Isolate Asp7 and Asp11 showed zone of activity 10 and 20mm respectively after the 15min UV exposure. The isolates Asp1, Asp3 and Asp 4 were unable to produce the zone of activity after the 15 min UV exposure.

Table 6 L-Asparginase production activity of isolates after 72 hours followed by exposure of UV after 5, 10 and 15 min respectively

Sr. No.	Code of isolates	L-Asparginase production after 5 min UV exposure	L-Asparginase production after 10 min UV exposure	L-Asparginase production after 15 min UV exposure
1	Asp1	10	12	0
2	Asp3	8	9	0
3	Asp4	7	8	0
4	Asp7	7	8	10
5	Asp11	12	20	26

The results of strain improvement for the production of L-asparginase after 72 hours of incubation were observed in table 7 and it was observed that the isolate Asp 1 were unable to produce the L-Asparginase after UV treatment of 15 min. and isolate Asp3 were unable to produce the L-Asparginase after UV exposure of 10 min. The Asp4 and Asp7 were not showed greater fluctuation in the production of the L-asparginase enzyme after UV treatment. Isolate Asp7 showed the 10 mm zone of activity after UV treatment for 15 min. The notable result of UV exposure was that the isolate Asp11 showed zone of activities as 20 and 26 mm after the exposure of 10 and 15 min respectively (Table 6)

Effect of pH on production of L-Asparaginase

Table 7 Effect of different pH on production of L-asparaginase after 24h incubation

Sr. No.	Code of Isolate	Zone of activity in mm on the M9 media plates with different pH				
		5	6	7	8	9
1	Asp1	8	12	15	14	0
2	Asp3	4	8	14	12	0
3	Asp4	6	10	14	13	0
4	Asp7	6	11	12	10	0
5	Asp11	4	14	18	14	8

The table 9 represents the effect of the different pH on the activity of the isolates to produce the L-asparginase. It was observed that the isolates showed 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 (Table 7).

Effect of temperature on production of L-Asparaginase

Table 8 Effect of different temperatures on production of L-asparaginase after 24 hrs incubation

Sr. No.	Code of Isolate	Zone of activity in mm on the M9 media plates with different temperatures in °C.				
		15	20	Room Temperature 25-30	37	45
1	Asp1	2	8	8	10	4
2	Asp3	0	2	6	8	6
3	Asp4	0	5	9	7	8
4	Asp7	4	6	4	7	0
5	Asp11	1	1	7	12	0

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 37^0C.

Purification of L-Asparginase Enzyme

The L-Asparginase obtained from the isolated species was purified by Ammonium sulphate precipitation (80% saturation), dialysis and CM-Sephadex C50 ion-exchange chromatography.

Table 9 Purification of L-asparginase Enzyme by series of methods

S N	Purification Step	Volume (ml)	Amylase activity (U)	Total protein (mg)	Specific Activity(U/mg)	Yield (%)	Purification (fold)
1	Crude extract	155	4530	355.80	12.73	100	1
2	Ammonium sulphate precipitation	53	3245	204	15.90	71	1.24
3	CM-Sephadex C50	34	2211	26	85.038	68	5.34

The steps for the purification were shown in the table 4. It was indicated that 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.

Electrophoresis (SDS-PAGE)

The purified enzyme was protein profiled by SDS PAGE for determination of molecular weight of the enzyme. The result thus obtained was a protein band of a molecular weight of approximately 45 kDa (Figure 10). Purified L-asparaginase from *Streptomyces gulbargensis* (Amena *et al*., 2010), *Streptomyces* sp. PDK2 (Dhevagi and Poorani, 2006) and *S. albidoflavus* (Narayana *et al*., 2007) exhibited a molecular weight of 85, 140 and 112 kDa, respectively. Reports on production and purification of L-asparaginase from *P. aeruginosa* revealed, by SDS- PAGE, a peptide chain with molecular weight of 160 kDa (El-Bessoumy *et al*., 2004).

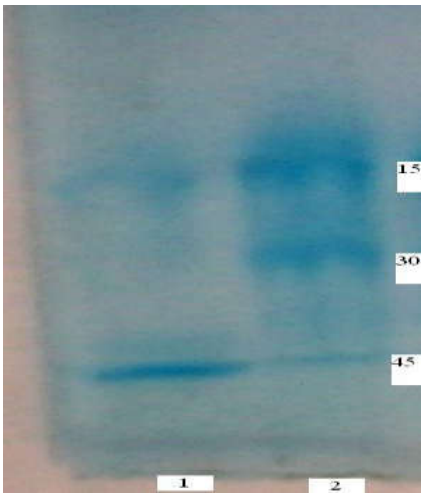

Fig 1 Purification and determination of the molecular weight of the L-Asparginase enzyme

Fig. 1 SDS PAGE-of L-asparaginase from Asp11 *Bacillus subtilis*

Electrophoresis was carried out on a polyacrylamide containing 0.1% SDS. The gel was stained with Coomassie blue. Lane 1 contained CM Sephadex C50 column purified enzyme. Lane 2 contains the standard markers. The molecular mass of enzyme was found to be 45 kDa. The Asp11 (*Bacillus subtilis*) highly potential species obtained from study can be used for the commercial production of the L-asparginase which is having immense medical applications in the treatment of the Leukemia.

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 UV radiation was found to be the easiest and the effective treatment for the strain improvement program. Strain improvement of the isolates for the enhanced production of the enzyme has given valuable information about the behavior of the isolates to UV treatment. Isolate Asp11 (*Bacillus subtilis*) was found to be the best candidate to

produce and to be used for the increased production of the enzyme. 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

Cornea CP, Lupescu I, Vatafu I, Caraiani T, Savoiu VG, Campeanu Gh, Grebenisan I, Negulescu Gh.P, Constantinescu D. (2002). Production of L-asparaginase II by Recombinant *Escherichia coli* cells. *Roumanian Biotechnology Letters*, 7(3):717-722.

De Jong P. J. (1972), L-Asparaginase production by Streptomyces griseus. *Appl. Microbiol*. 23, 1163 -1164.

El-Bessoumy A A, Sarhan M, Mansour J. (2004). Production, isolation, and purification of L-Asparaginase from Pseudomonas aeruginosa 50071 using solid-state fermentation. *Journal of Biochemistry and Molecular Biology*, 37(4):387-393.

Holt JG, Krieg RN, Sneath PHA, Staley JT, Williams ST. (2000). Bergey's manual of determinative Bacteriology. 9thedn. Lippincott Williams and Wilkins, Baltimore, Maryland, USA.

Kafkewitz D, Goodman D. (1974). L-asparaginase Production by the Rumen Anaerobe Vibrio succinogenes. *Applied & Environmental Microbiology*, 27(1): 206-209.

Mashburn LT, Wriston JC. (1964). Tumor Inhibitory Effect of L-asparaginase from Escherichia coli. *Archives of Biochemistry and Biophysics,* 105:450-452.

Moorthy, V., Ramalingam, A., Sumantha, A., Shankaranaya, R.T. (2010). Production, purification and characterisation of extracellular L- Asparaginase from a soil isolate of Bacillus sp. *Afr. J. Microbiol. Res*., 4: 1862-1867.

Parekh S., Vinci VA. and Strobel RJ. (2000). Improvement of microbial strains and fermentation processes. *Appl Microbiol Biotechnol* 54: 287

Peterson R. E. and Ciegler A. (1969), L-Asparaginase production by various bacteria. *Appl. Microbiol*. 17, 929 - 930.

Prakasham, R.S., Hymavathi, M., Rao, C.S., Arepalli, S.K., Rao, J.V., Kennady, P.K., Nasaruddin, K., Vijayakumar, J.B., Sarma, P.N. (2010). Evaluation of Antineoplastic Activity of Extracellular Asparaginase Produced by Isolated Bacillus circulans. *Appl. Biochem. Biotechnol.* 160: 72-80.

Sarquis MIdeMoura, Oliveira EMM, Santos AS, da Costa GL. (2004). Production of L-asparaginase by filamentous fungi. Memórias do Instituto Oswaldo Cruz, 99(5): 489-492

Sinha, A., Manna, S., Roy, 5. K. & Chakrabarty, 5. L. (1991). Induction of L-asparaginase in Vibrio protezts. *Indian J Med Res* 9, 289-292.

Tosa T, Sano R, Yamamoto K, Nakamura M, Ando K, Chibata I. (1971). L-Asparaginase from Proteus vulgaris. *Applied & Environmental Microbiology*, 22(3): 387-392

Wrismn, J. C. (1970). Asparaginase. Methods in Enzymology 17A, 7 32-742.

How to cite this article:

Patil R.C and Jadhav B. L.2017, Improvement of Bacillus Species By Mutation For Production of L-Asparginase. *Int J Recent Sci Res.* 8(6), pp. 17698-17702. DOI: http://dx.doi.org/10.24327/ijrsr.2017.0806.0407

****