ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation and Characterization of Bacteria from Coal Field Slurry for L-Asparginase Production in *In-vitro* Condition.

Anupam Kumar*, Chetna Thakur, Smriti Sharma, Bhawna Sharma, Aman Agrawal, and Gopal Kumar Roy.

Lovely Professional University, Phagwara, Punjab, India.

ABSTRACT

L-asparaginase is one of the most demanding enzyme for various significant role like anti- tumour activity and useful in acute lymphoblastic leukaemia, lymph sarcoma and an application in the food industries. It is an enzyme that reduces the activity of Asparagine because it is the nutritional requirement for both tumour and normal cell. It is characterized on the basis of enzyme assay principle hydrolysing L-asparagine into L-aspartic acid and ammonia. High yields of L-asparaginase enzyme and its optimization process is always targeted by bacterial sources, in current study L-asparginase producing bacteria were isolated and characterized, soil samples were collected from the coal slurry of coal field, Dhanbad, Jharkhand. For the isolation of L-asparaginase, the soil sample was inoculated in enrichment media and incubated for 24 hours .After spreading of the sample the colonies were picked randomly and streaked on agar slants and incubated for 24 hours. Qualitative screenings of eight isolates for L-asparaginase production were done on Bromothymol blue plates. At first the eight isolates, were point inoculated on Bromothymol blue plates and three producers were selected and one of the isolates was characterized and optimization parameters were analyzed.

Keywords: L-asparaginase, Coal slurry, Bromothymol blue, Qualitative screening, Optimization

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Bacterial type L-asparaginases are classified into subtypes I and II, which are defined by their intra- or extracellular localization [3]. Type I (Cytosolic) has a lower affinity for L- asparagine and type II (Periplasmic) has a high affinity for L-asparagine. Plant type L- asparaginases differ structurally and have a different evolutionary origin than bacterial type L-asparaginase [3]. The enzyme L-asparaginase is an anti-tumour agent for the treatment of acute lymphoblastic leukaemia and also lymph sarcoma [1, 15]. It's characterized on the basis of enzyme assay principle hydrolysing L-asparagine into L-aspartic acid and ammonia [2]. L-asparagine is a majorly required by the cells for the production of protein. It can be produced within the cell by an enzyme called asparagines synthetase or can be absorbed from outside. For the growth of tumor cells L-asparagine is an essential amino acid [5, 8]. The growth of tumor is inhibited by L-asparaginase by degrading the asparagines present in tumour cell [10]. The activity of enzyme at the alkaline pH range at 37^0C make it extremely valuable in the chemotherapeutic treatment of leukaemia [7]. L- Asparaginase has given complete remission in some human leukaemia. It can be used in food industries as a food processing aid to reduce the formation of acrylamide in starchy food products when they are baked or fried [13]. L-asparagine is converted into another common amino acid, aspartic acid, and ammonium by adding asparaginase before frying the food as a result formation of acrylamide is significantly reduced [5].

MATERIAL AND METHODS

Sample collection

Soil samples were collected in a sterilized polythene bag from the coal slurry. L-asparginase producing bacteria were isolated from coal slurry near coal mines and coal industry of Dhanbad, Jharkhand containing waste from the coal processing industries, coal washrey, oil refinery industries and grease.

Isolation of microorganisms producing L-asparaginase

Isolation procedure: To isolate bacteria producing L-asparaginase from the soil sample the following procedure was followed:

Direct plating method: Different samples were directly plated onto nutrient agar plates, after serially diluting samples in autoclaved distilled water using dilution plate method. Then inoculated samples were kept at 37°C for 24 hours in bacteriological incubator. The composition of Nutrient agar medium (pH – 7.2 ± 0.2) Peptone 5.0g/L Beef extract 3.0g/L NaCl 8.0g/L Agar-Agar 20g/L.

Preparation of the medium: All the above composition were poured in 1000ml of distilled water and the pH was adjusted to 7.0 using 1N HCL or 1N NaOH. The medium was autoclaved for 15 minutes at 15 psi. This sterilized medium was poured into Petri dishes to make nutrient agar plates or in test tube for slant.

Culture enrichment technique

Composition of enrichment media: Peptone 5g/L, Beef extract 5g/L, Glucose 12g/L, KH_2P 3g/L, KH_2PO_4 1g/L. The above ingredients were poured in 200 ml of distilled water and the pH was adjusted at 7.0. Then the media was divided into 50 ml each in 4 flasks. The media was autoclaved at 15 psi at 121 C. Then isolation of L-asparaginase producers was carried out by inoculating 1 gm of soil sample in 50 ml of media flask. Flask was then kept for an incubation period of 24 hours at 37^0C and 200 rpm in the incubator shaker, to allow micro- organisms present in the soil to grow into vegetative form. [9]

After 24 hours, the sample was diluted by serial dilution from 10^{-3} to 10^{-8} and each dilution was inoculated on agar plates using spreading technique to isolate colonies and incubated at 37^0C for 24 hours in BOD incubator. Eight colonies from spreading plates were picked randomly and streaked on agar slants and incubated in BOD incubator at 37^0C for 24 hours.

ISSN: 0975-8585

Production medium for the L-asparaginase producing microorganisms:-

The 5 different medium for L-asparaginase producers were prepared and then media were inoculated with the loop full of cultures and incubated at 37^{0}C at 200 rpm and the growth in all medium were checked by taking ODs at 24 hours and 48 hours to find out the best production media for the producers of L-asparaginase [11][13].

The composition of 5 different medium is given below:

Ingredients	g/l	Ingredients	g/l	Ingredients	g/l	Ingredients	g/l	Ingredients	g/l
$Na_2HPO_4.2H_2O$	6	KH2PO4	2	K_2HPO_4	1	$NaNO_3$	40	Glucose	2
KH_2PO_4	3	$MgSO_4.7H_2O$	1	Glucose	1	KCl	10	KH2PO4	1.52
NaCl	0.5	$CaCl_2.2H_2O$	1	Yeast extract	5	$MgSO_4.7H_2O$	0.52	KCl	0.52
L-asparagine	5	L-asparagine	6	Tryptone	5	$FeSO_4.7H_2O$	0.2	L-asparagine	10
$MgSO_4.7H_2O$(1M)	2 ml/l	Glucose	3			K_2HPO_4	20	$MgSO_4.7H_2O$	0.52
$CaCl_2.2H_2O$(0.1M)	1 ml/l					$ZnSO_4.7H_2O$	1	$Cu(NO_3)_2.3H_2O$	Trace
Glycerol (20%)	10 ml/l					$CuSO_4.7H_2O$	0.5	$FeSO_4.7H_2O$	Trace
						Glucose	30	$ZnSO_4.7H_2O$	Trace

Identification of the isolated bacterial cultures

Biochemical and morphological characterization of isolated bacterial cultures were performed on the basis of their growth characteristics on different differential media and by performing staining techniques like Gram and negative staining.

Qualitative screening of L-asparaginase producing microorganisms

Qualitative screening of eight isolates for L-asparaginase production was done on Bromothymol blue plates. At first the eight isolates, were point inoculated on Bromothymol blue plates and three producers were selected, as in these three zone of hydrolysis was observed [12].

L-asparaginase enzyme characterization

Purified form of L-asparaginase provided by laboratory stock culture collection was evaluated and compared for different properties.

Formulae's for calculation of Enzyme activity of L-asparaginase

$$\mu\text{moles of Ammonia released} = \left(\frac{O.D. - 0.0046}{0.0947}\right) \times 2$$

Where O.D. = Optical Density

O.D. = O^{D}_{T} - O^{D}_{C} Where O^{D}_{T} = OD of Test

O^{D}_{C} = OD of Control

$$\textbf{Enzyme Activity} = \left(\frac{\mu\text{moles of Ammonia released} \times 2}{0.2 \times time \times 0.1}\right) \times \text{Dilution Factor}$$

Where 2.0 = Total enzyme reaction

0.2 = Enzyme reaction evaluate

0.1 = Enzyme volume taken

Effect of temperature on L-asparaginase activity: Temperature tolerance of L- asparaginase was studied by incubating L-asparaginase sample with L-asparaginase at different temperatures 10°C, 25°C, 30°C, 37°C and 45°C.The optimum temperature of L- asparaginase activity was found to be 37^{0}C. It is

ISSN: 0975-8585

active at a wide range of temperature condition from 37^0C to 45^0C. It shows an increase in enzyme activity from 10^0C to 37^0C and gradually decline at 45° C. [4]

Effect of pH on L-asparaginase activity: L-asparaginase enzyme pH tolerance at different pH was studied by incubating the L- asparaginase sample with L-asparagine in buffers of different pH (4-10) [14].

RESULTS AND DISCUSSION

Screening of L-asparaginase producing microorganisms

Screening is done by following method:

1. Qualitative screening
2. Quantitative screening

Qualitative screening of L-asparaginase producing microorganisms

Qualitative screening of eight isolates for L-asparaginase production was done on Bromothymol blue plates. At first the eight isolates, were point inoculated on Bromothymol blue plates and three producers were selected.

Screening on Bromothymol blue plates after 24 hours of incubation:-

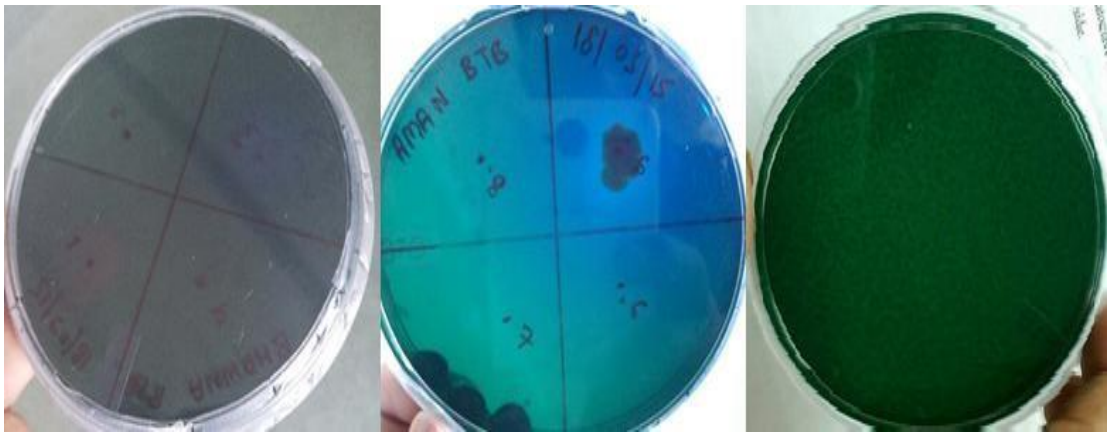


Figure 1: L-asparginase production is seen in 1, 3 and 5 Control

Screening on Bromothymol blue plates after 24 hours of incubation

Table 1: Isolates vs asparginase production

Isolate number	L- asparaginase production
1	+ + +
2	- -
3	+ + +
4	- -
5	+ + +
6	- -
7	- -
8	- -

ISSN: 0975-8585

Biochemical characterization

Table 2: Biochemical characterization

Biochemical tests	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5
Gram staining	+ve	+ve	+ve	-ve	-ve
Catalase	+ve	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	+ve	+ve
Xylose	+ve	+ve	-ve	-ve	-ve
Glucose	+ve	+ve	+ve	-ve	+ve
Maltose	+ve	+ve	+ve	-ve	-ve
Fructose	+ve	+ve	+ve	-ve	+ve
Mannitol	+ve	+ve	+ve	-ve	-ve
Sucrose	+ve	+ve	+ve	-ve	+ve

The best producer of L-asparaginase was selected out of 5 strains i.e., isolate no. 5 and quad streaked on agar plates.

Figure 4: Streaking of sample 5

Staining of isolated sample no. 5

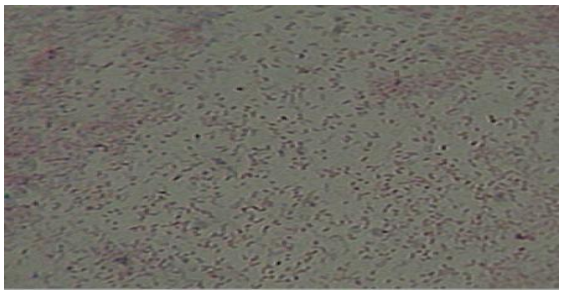


Figure 5: Sample 5 Gram staining

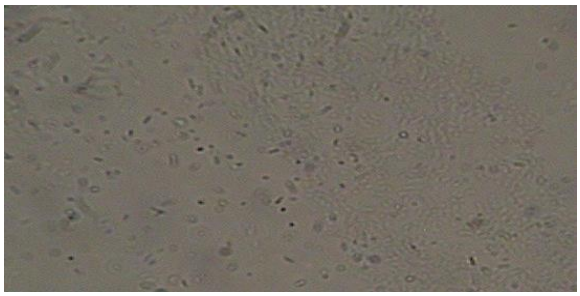


Figure 6: Sample 5 Negative staining

Colony morphology of isolate no.5:-

Table 3: Colony morphology

Characteristics	Isolate no.5
Form	Circular
Size	Large
Texture	Dry
Colour	Colourless
Opaqueness	Opaque
Elevation	Umbonate

ISSN: 0975-8585

Quantitative screening

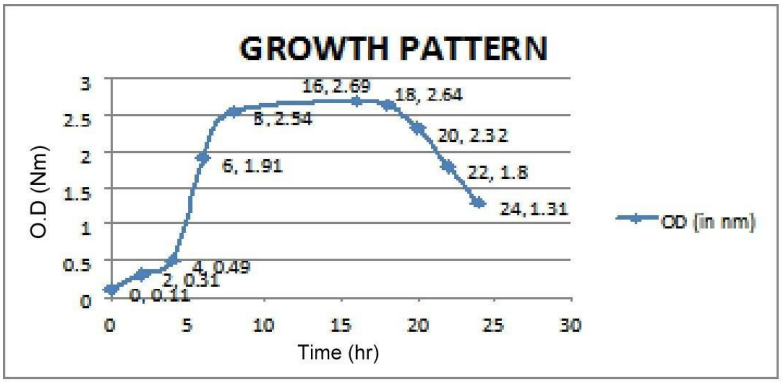
Growth curve of L-asparaginase producing microorganisms

Growth curve shows the growth pattern of microorganisms including all the phases i.e., lag phase, exponential or log phase, stationary phase and decline/death phase. The sample was inoculated in the nutrient broth and the samples were withdrawn after every 2 hours of incubation at 37^0C 200 rpm. The results obtained after sample withdrawal are presented in the following table:

Growth curve of L-asparaginase producing microorganisms:-

Table 4: OD at different time (hours)

Time	OD
0	0.11
2	0.31
4	0.49
6	1.91
8	2.54
16	2.69
18	2.64
20	2.32
22	1.8
24	1.31



Graph 1: It is revealed by the above graph that maximum growth of the culture of isolate no. 5 is seen at 16th hour.

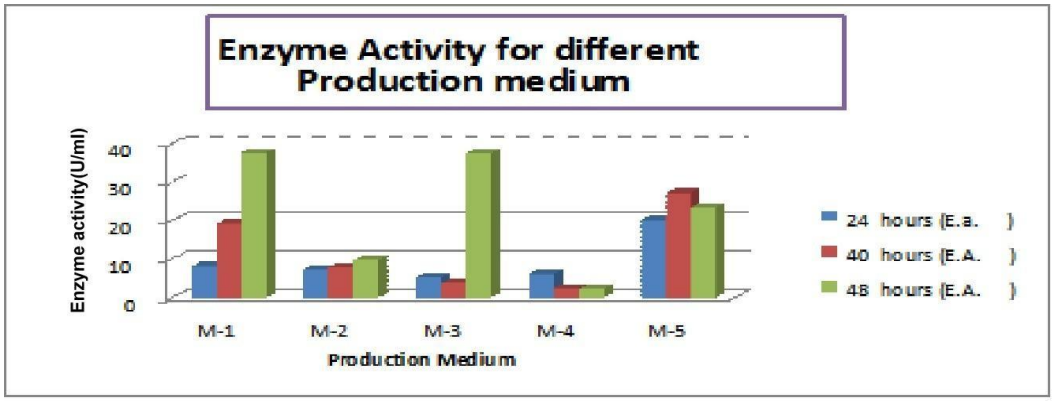
The observations made for different production medium for isolate no. 5 are as follows:

Production medium for isolate no. 5:-

Table 5: Production medium

Production media	Enzyme Activity (24 hours)	Enzyme Activity (40 hours)	Enzyme Activity (48 hours)
M-1	8.13	19.1	37.53
M-2	7.43	7.9	9.67
M-3	5.3	4.7	7.33
M-4	6.15	2.36	2.1
M-5	20.07	27.1	23.33

ISSN: 0975-8585



Graph 2: Production medium of isolate no. 5

The enzyme activity was calculated for different for isolate no. 5 for 24, 40 and 48 hours. The best optimum production medium for isolate no. 5 was found to be Media 5.

Effect of Physical parameters on L-asparaginase activity

Effect of temperature

Temperature tolerance of L-asparaginase was studied by incubating L-asparaginase producing sample at different temperatures 10°C, 25°C, 30°C, 37°C and 45°C.

Effect of pH on L-asparaginase activity

L-asparaginase enzyme pH tolerance at different pH was studied by incubating the L-asparaginase sample with L-asparagine in buffers of different pH (4-10).

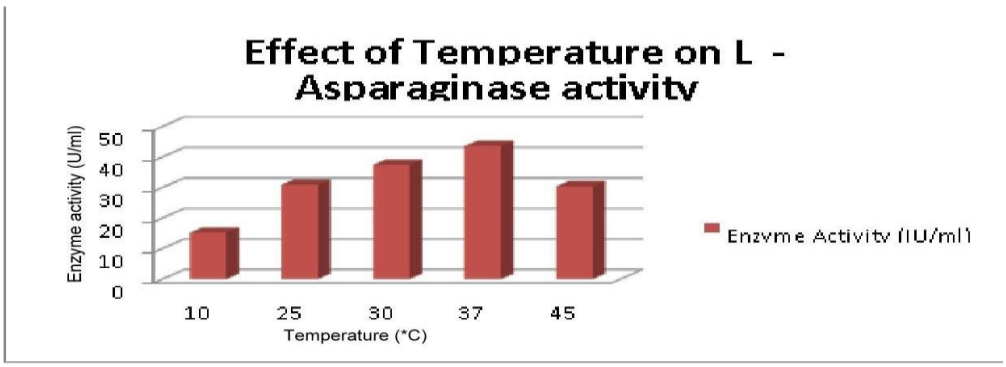
Table 6:- a and b

Effect of temperature on L-asparaginase activity

Temperature	Enzyme Activity (IU/ml)
0	15.2
5	30.93
30	37.4
37	43.6
45	30.4

Effect of pH on L-asparaginase activity

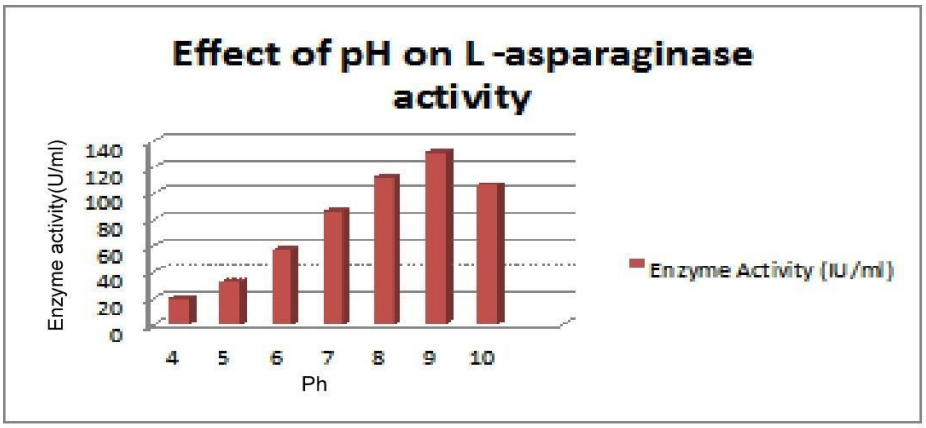
pH	Enzyme Activity (IU/ml)
4	20.08
5	32.4
6	56.7
7	86.8
8	112.6
9	131.08
10	106.4



Graph 3: Effect of temperature on Enzyme activity

ISSN: 0975-8585

The optimum temperature of L-asparaginase activity was found to be 37 °C. It is active at a wide range of temperature condition from 10 °C to 45 °C. It shows an increase in enzyme activity from 10 °C to 37 °C and gradually decline at 45 °C.



Graph 4: Effect of pH on L-asparaginase activity

It is evident from the results presented in the table and graph that L-asparaginase was active over broad range of pH (4-10) with an optimum pH 7. The relative activity gradually showed an increase from pH 4 till pH 9 which is found to be optimum and then it decline at pH 10.

CONCLUSION

The isolated colonies obtained after spreading on the nutrient agar plates, were selected and their morphological characteristics were studied. The colonies were streaked on nutrient agar plates using quadrant streaking method. The streaked colonies were point inoculated on modified medium supplemented with Bromothymol Blue or Phenol red. The change in colour of the medium due to ammonia accumulation indicates L-asparaginase production. Process optimization by one variable at a time approach was followed. According to the results obtained the optimum pH was 9.0 optimum temperatures was 37°C with 200 RPM agitation.

L-asparaginase is one of the important anti-tumour enzymes, further a detailed biochemical, molecular characterization and industrial production can be done for specific isolate and new resources can be targeted for enzyme production.

ACKNOWLEDGEMENT

Authors are thankful to Lovely Professional University and all concerned authorities for providing the all facilities and provisions during completions of research work.

REFERENCES

[1] Yadav Soni, Verma Kumar Sitanshu, Singh Jitendra, Kumar Ajay. International Scholarly and Scientific Research & Innovation 2014; 1: 54-60.
[2] Jain Richa, Zaidi KU, Verma Yogita, Saxena Pooja. People's Journal of Scientific Research 2012; 1: 29-35.
[3] Michalska Karolina, Jaskolski Mariusz. Acta Biochimica Polonica 2006; 53: 627-640.
[4] El-Sabbagh Mahmoud Sabha, El-Batanouny Hamed Nadia, Salem A. Tarek. African Journal of Microbiology Research 2013; 7(50): 5677-5686.
[5] Dhanam Jayam G, Kannan S. International Journal of Recent Scientific Research 2014; 2: 342-346.
[6] Gulati R, Saxena RK, Gupta R. Letter to Applied Microbiology 1997; 24: 23-26.
[7] Shah AJ, Karadi RV and Parekh PP. Asian Journal of Biotechnology 2010; 3: 169-177. [8]
[8] Kumar Kuldeep, Verma Neelam. Asian Journal of Biochemical and Pharmaceutical Research 2012; 3: 197-205.

[9] Kirk Ole, Borchert Vedel Torben, Fuglsang Crone Claus. Current Opinion in Biotechnology 2002; 13: 345–351.
[10] Savitri, Asthana Neeta, Azmi Wamik. Indian Journal of Biotechnology 2002; 2: 184-194.
[11] Basha NS, Rekha R, Komala M, Ruby S. Tropical Journal of Pharmaceutical Research 2009; 4: 353-360.
[12] Verma N, Kumar K, Kaur G, Anand S. Critical Review in Biotechnology 2007; 27:45-62.
[13] Pedreschi F, Kaack K, Granby K. Food Chemistry 2008; 109: 386-92.
[14] Warangkar SC, Andkhobragade CNAM. J. Biochem. Biotechnol 2008; 4: 422-424.
[15] Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A. US National Library of Medicine 2011; 2: 238–249.