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Screening and Optimization of L– Asparginase Produced by Bacteria Isolated from Soil of Solapur Region

Afshaan Naaz Shaikh Khaleed[1], R. M. Khobragade[2]

Department of Microbiology, Dr. Babasaheb Ambedkar Marathwada University, Sub-Campus, Osmanabad.(MS).413501

Abstract: ***L-asparaginase (E.C No 3.5.1.1) is a potential anti-carcinogenic enzyme. Seven different soil samples from Solapur regions were screened for potential producers of L-asparaginase using modified Czapek dox's agar containing L-asparagine and phenol red as indicator with pH 7. The strain isolated from slaughter house soil sample (S-2) showed the maximum enzyme activity. The enzyme production was carried out by submerged fermentation. The maximum enzyme activity was 103.7 U/ml was showed by 24 hrs old culture at 37° C.***

Keywords: L-asparaginase, anticancer, bacteria

1. Introduction

L-asparagine is an essential amino acid used by both normal cells and cancer cells. L-asparaginase enzyme cleaves L-asparagine into aspartic acid and ammonia. Many types of cancerous cells require L-asparagine for protein synthesis they are deprived of an essential growth factor in the presence of L- asparginase. This enzyme is mainly used in the treatment of acute lymphatic leukemia (ALL) in children's [1, 2]. These tumor cells require large amount of L-asparagine for malignant growth. [3]

$$\text{L-asparagine} + H_2O \xrightarrow{\text{L-asparaginase}} \text{Aspartic acid} + \text{Ammonia } (NH_3)$$

L-asparaginase was produced by wide range of algae, bacteria, actinomycetes, fungi, and plants [4]. Microbes are easily cultured and their products are easily extracted, purified and methods are also convenient, for this reason they are better source for the production of L-asparginase[5]. L-asparaginase creates antineoplastic activity results from depletion of the circulating pools of L-asparagine[6] .This will result in inhibition of DNA and RNA synthesis with subsequent blastic cell apoptosis[7]. Owing to the unique anti cancer mechanism of action, L-asparaginase has been introduced to the multi drug chemotherapy in children and adults with acute lymphoblastic leukemia (ALL), which has contributed to significant improvement of therapy outcomes and to achieve complete remission in approximately 90 % of patients[8]. The objective of present research study was to isolate the potent bacterial strain producing large amount of L-asparaginase with maximum activity.

2. Materials and Methods

Chemicals

All chemical used in this research work were of analytical grade were purchased from Himedia, Mumbai, India.

Collection of Samples

Seven soil samples were collected from different places at a depth of 5 to 10 cm in and around the Solapur regions, Maharashtra, India. The samples were collected into a sterile polythene bags and carried to departmental research lab for further study. Samples were stored at 4°C, till further use.

Isolation of Bacteria

Different soil samples were used for bacterial isolation by serial dilution method, 10^{-4} and 10^{-5} dilutions were used and spread on nutrient agar medium. To avoid the fungal contamination nystatin (50µ g/mL) added to the medium. The inoculated agar plates were incubated at 37° C for 24 hrs. Isolated colonies were transferred to slant and stored at 4°C.

Screening of L- Asparginase By Plate Method Assay

The isolated bacterial colonies were screened for L-asparaginase production on modified Czapek dox agar containing glucose-2.0, Lasparagine-10.0, KH_2PO_4-1.52, KCL-0.52,$MgSO_4.7H_2O$-0.52, $CuNO_3.3H_2O$-trace, $ZnSO_4.7H_2O$-trace, $FeSO_4.7H_2O$-trace (grams per liter of distilled water) at pH 7 and 0.09%(v/v) phenol red as a indicator. Strain with pink colour zones around the colonies were considered as L-asparaginase producing strains. Development of pink colour zones around the colonies was considered as positive result for L-asparaginase production.[9]

Enzyme Production By Submerged Fermentation Method

L-asparaginase production was carried out by submerged fermentation[10]. A 250mL Erlenmeyer flask containing 50mL of sterilized medium was used for production. A loop full of 24 hour old culture was inoculated into the above said medium. The flask was placed in an incubating orbital shaker at 120 rpm at 37° C for 24 hrs. Un-inoculated medium served as control. The bacterial cell mass was separated by centrifugation at 5000 rpm for 15 min at 4° C. The liquid

supernatant was used as crude enzyme source to determine the enzyme activity.

Estimation of L-Asparaginase Enzyme Activity
L-asparaginase activity was carried out by using Mashburn and Wriston determination method[11]. The reaction mixture containing 0.5 ml of 0.04 M L-asparagine, 0.5 ml of 0.5 M phosphate buffer (pH 7.8), 0.5 ml of an enzyme and 0.5 ml distilled water was added to make up the total volume to 2 ml. The tubes were incubated at 30°C for 30 minutes. The reaction was stopped by adding 0.5 ml of 1.5 M Trichloroacetic acid (TCA). The blank was prepared by adding enzyme after the addition of TCA. To the 3.7 ml of distilled water and 0.2 ml of Nessler's reagent, 0.1 ml from the above mixture was added. After incubating the mixture at 20°C for 20 minutes the OD was checked at 450 nm with Spectrophotometer. The enzyme activity was expressed in International unit.

Optimization of Parameter for Higher Production of L-Asparginase.
The asparaginase production of potent isolate was optimized under shake flask culture. The effects of different parameters including batch time, inoculum size, pH and temperature on enzyme production were studied and asparginase activity analysed by standard asparginase assay.

- **Primary inoculum preparation:**

For inoculum preparation, a loop full of 24 hr old pure culture of selected isolate was transferred in 20 ml of sterile modified Czapek dox's medium and flask was incubated overnight at 37°C in a rotary shaking incubator at 180 rpm.

1. Effect of Fermentation Time:
Optimum fermentation time for asparaginase production was determined by determining enzyme activity using modified Czapek dox's medium at different time intervals from 24h to 144h with a variation of 24h, flasks were incubated at 37 ºC with shaking at 180 rpm.

2. Effect of Inoculum Size:
Different inoculum size for maximum asparaginase production was determined by determining enzyme activity using modified Czapek dox's medium at different inoculum size(5%,10%,15%,20%,25%,30%), flasks were incubated at 37 ºC with shaking at 180 rpm.

3. Effect of Different temperature:
Enzyme activity was detected at different temperatures (20, 30, 37, 40, 45 and 50°C) by using modified Czapek dox's medium and flasks were kept at 180 rpm.

4. Effect of Different pH:
Enzyme activity was detected at different pH ranges from 6 to 10, by using modified Czapek dox's medium and flasks were incubated at 37 ºC with shaking at 180 rpm.

3. Results and Discussion

Isolation of Bacteria
In the present study, 49 different bacterial cultures were isolated from soil samples, collected from seven different places in and around the Solapur regions, Maharashtra, India. Potent isolates were labeled as BSH, BAF,BD ,BG, BPP, BCS, BM(Table 1) .

Screening of L- Asparginase By Plate Method Assay
The isolated strains were screened using modified Czapek Dox medium supplemented with phenol red for L-asparaginase production. The active asparaginase producing bacterial colony changed the colour of the medium from yellow to pink. This colour change was due to change in the pH of the medium, as L-asparaginase causes the breakdown of amide bond in L-asparagine and librated ammonia [12]. From the 49 bacterial isolates, 7 colonies were positive for extracellular L-asparaginase production. The remaining isolates, as discerned by plate assay, were non-producers. Positive seven isolates were tested quantitatively for L-asparaginase activity.

Estimation of L-Asparaginase Enzyme Activity
The asparaginase producing strains were morphologically studied (Table 2). Out of seven isolates BSH-1 showed maximum enzyme production (103.7U/ml) and the isolate BPP5 exhibited minimum enzyme production (29.3U/ml) (Table 3).. Similarly Thirunavukkarasu N[13] have reported that maximum activity was obtained at 24 hours of incubation from the bacterial isolates. Ellaiah[14] have reported that growth rate and enzyme synthesis of the culture are the two main characteristics which are mainly influenced by incubation time. In the present study, the maximum enzyme production by BSH-1 was obtained after 48 hours, (103.7 U/ml).

Optimization of Parameter For Higher Production of L-Asparginase.
(BSH-1 - High producer) was chosen to study the effects of incubation time, pH, temperature and inoculum size on enzyme production.

1. Effect of Fermentation Time
In general many enzymes are active at the range of 24-72 h. Maximum enzyme production could be obtained only after a certain incubation period which allows the culture to grow at a steady state [15]. In this present research work the maximum enzyme production by BSH-1 was obtained after 48hrs , (103.7 U/ml) .

2. Effect of Inoculum Size
Effect of different inoculum size was tested on maximum production of enzyme, optimum enzyme production observed at 10% inoculum size. This data further confirmed that fermentation factors and their concentrations were important in achieving better enzyme production. Gupta *et al.* 2003[16] and Prakasham *et al.* 2005a[17] was also noticed such variation with enzyme production by other microbes.

3. Effect of Different temperature
In the present study, incubation temperature influenced the production of L- asparaginase by BSH-1. Manna [18] have reported 37°C as the optimal temperature for maximum activity by *Pseudomonas stutzeri*. In the present study, maximal L- asparaginase production was observed at 37°C (103.7 U/ml) and minimum production was at 45°C (21 U/ml). Further increase in temperatures adversely affected

the enzyme production. Sarqius [19] have reported 30°C is suitable for L-asparaginase production through submerged fermentation by using *A.terreus* and *A. tamari.*

4. Effect of Different pH

In the present study, maximal enzyme production was observed at pH 7 (103.7 U/ml) and pH above and below pH 7 led to a decrease in L- asparaginase production. The minimum enzyme production was observed at pH 10 (19 U/ml). Similar results have been reported by De-angeli [20] that pH 7.0 is the optimum pH for L-asparaginase production under submerged fermentation process.

Table 1: Isolation and screening of L-asparaginase producing bacteria from different sites

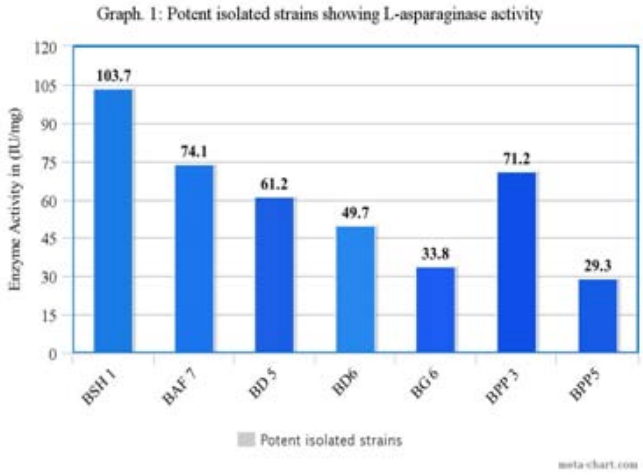
Source of soil samples	*Sample code*	*Total no of isolates*	*No of potent isolates showing L asparaginase*
Mill	BM	4	00
Construction site	BCS	7	00
Slaughter house	BSH	2	01
Agriculture field	BAF	12	01
Dairy	BD	8	02
Garden	BG	11	01
Petrol pump	BPP	5	02

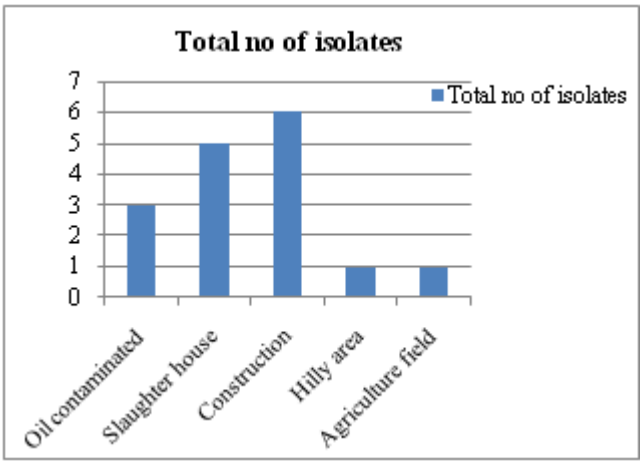
Table 2: Morphology and cultural characteristics of L-asparaginase producing bacterial isolates

Sample code	*Cultural Characteristics*	*Grams Nature*
BSH-1	**Yellowish, circular, slimy colonies**	Gram –ve bacilli
BAF-7	White, irregular, oval shaped colonies	Gram –ve bacilli in clusters
BD-5	Minute lemon yellow colonies	Gram +ve bacilli
BD-6	Orange, circular, sticky colonies	Gram –ve cocci
BG-9	yellow, minute colonies	Gram +ve cocci in clusters
BPP-3	Minute, white, irregular	Gram –ve bacilli
BPP-5	Large, yellowish, rough irregular colonies	Gram –ve cocci in pairs

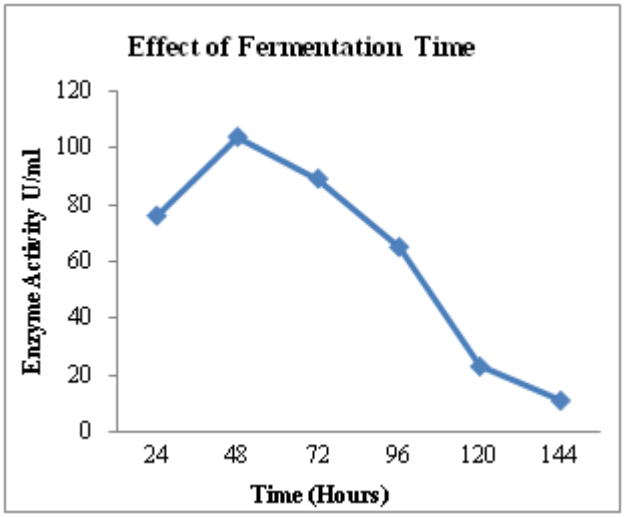
Table 3: Bacterial isolates showing L-asparaginase activity

Sample code		*L-asparaginase activity (IU/mg)*
BSH	1	103.7
BAF	7	74.1
BD	5	61.2
	6	49.7
BG	6	33.8
BPP	3	71.2
	5	29.3

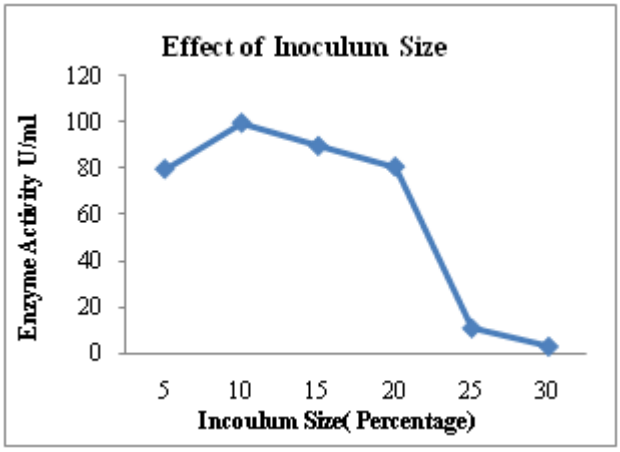




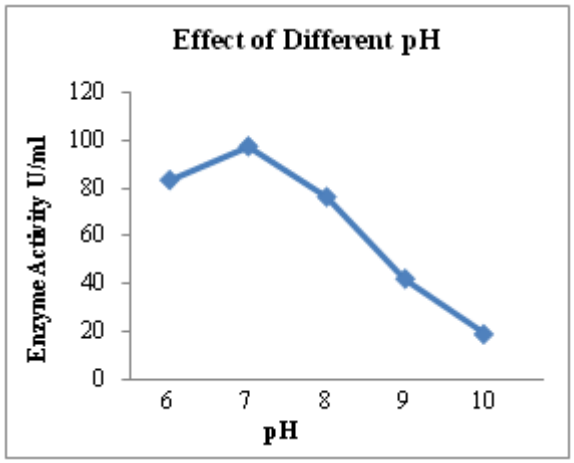
Graph 2: Isolates showing positive results



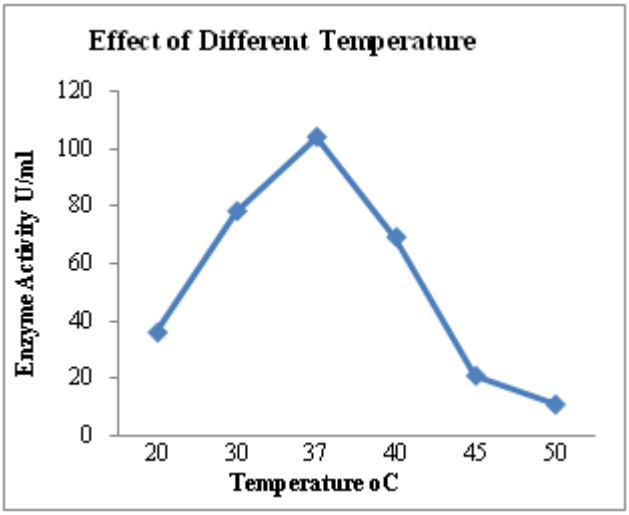
Graph 3: Effect of fermentation time on Asparginase Activity



Graph 4: Effect of Inoculum Size on Asparginase Activity



Graph 4: Effect of Different pH on Asparginase activity



Graph 5: Effect of Different Temperature on Asparginase activity

4. Conclusion

From this present research work, it was showed that slaughter house, agricultural filed and petrol pump site's soil samples can provide a rich source of L-asparaginase producing bacteria when compared to other soil samples. It was also indicated that dairy industry soil sample show very low L-asparaginase producing bacteria, however mill and construction site's soil sample does not show L-asparginase producer. Further this study reveals that maximum production of L-asparaginase enzyme after optimization of fermentation parameters such as fermentation time, temperature, pH and inoculum size. This study indicates that the BSH-1 is a potential strain for L-asparaginase production and 37°C temperature at 7pH is the optimum condition for the production of L-asparaginase enzyme. Maximum enzyme production was observed after 48 hours by using 10% inoculum. In future study the BSH-1 bacteria will be identified up to genus and species level. Additional parameter will be considered for maximum production of enzyme L-asparginase.

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