Research Paper

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# Studies on L-Asparaginase Producing Bacteria from Soil Samples of Akola District

Microbiology

**KEYWORDS :** L- asparaginase, Bacillus, P. aeruginosa, Serratia,

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## ABSTRACT

*L- Asparaginase is an extracellular enzyme having potential therapeutic applications. The present work describes the isolation, screening, Identification, enzyme production, protein estimation and characterization of bacteria isolated from various parts of Akola region. Bacteria were screened for L- asparaginase production from farm soil on the basis of formation of pink color zone around the colony. L-asparaginase producing bacteria were further characterized by morphological, physiological and biochemical studies as Bacillus sp, P. aeruginosa, Serratia sp. and S. aureus. Maximum growth was observed at optimum conditions (pH 7, temperature at 37°C and time 72 rrs.) Maximum protein estimated from isolate no. SR 9. The crude enzyme characterized for effect of temperature, pH, metal ion and substrate and enzyme concentration.*

## INTRODUCTION :-

L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) was introduced in the therapeutics to treat acute lymphoblastic leukemia. However, L-asparaginase is of special significance because of the fact that tumour cells are deficient in L-asparaginase synthetase activity, which restricts their ability to synthesize the normally non-essential amino acid L-asparagines, required for the growth and survival of cancer cells. Therefore, tumour cells are dependent on exogenous supply of L-asparagines from body fluids. Administration of L-asparaginase within the body does not affect the functioning of normal cells because they possess an inherent property to synthesize L-asparagines for their own requirements, but reduces its concentration in the plasma pool. Thus induces a state of fatal starvation in the susceptible tumour cells L-asparaginase is an important therapeutic enzyme and is produced by bacteria (Upadhyay et al., 2012).

The most common use of L-asparaginase is as a processing aid in the manufacture of food. Marketed under the brand names Acrylaway and PreventASe L-asparaginases are used to reduce the formation of acrylamide, a suspected carcinogen, in starchy food products such as snacks and biscuits. A different L-asparaginase is marketed as a drug under the brand name Elspar for the treatment of acute lymphoblastic leukemia (ALL) and is also used in some mast cell tumor protocols. Unlike other chemotherapy agents, it can be given as an intramuscular, subcutaneous, or intravenous injection without fear of tissue irritation. The exact mechanism of its action is still unknown although hydrolysis proceeds in two steps via a beta-acyl-enzyme intermediate (Kushwaha et al., 2012).

The medical utilization of L-asparaginase from the reported sources suffer the limitations of eliciting immunological responses leading to hypersensitivity in the long-term usage, allergic reactions, anaphylaxis and instance of spontaneous resistance of the tumor cells. So the present investigation was undertaken with an aim to search for some excellent L-asparaginase producer and study optimization criteria for the production of cost effective, eco-friendly and a potent L-asparaginase in near future.

## MATERIAL AND METHODS :-

### ➢ Isolation, Identification and Screening of Maximum L-Asparaginase Producers:-

16 soil samples were collected in sterile ziplock polythene bags from various parts of Akola region and then transferred to laboratory for further study.

L-asparaginase producing microorganisms were isolated from collected soil by serial dilution of soil and spreading on agar plates. Mixed cultures were obtained after incubation and obtained mixed cultures were purified by streaking procedure on agar plates.

Screening for maximum L-asparaginase producer was done by primary and secondary screening technique. The primary screening was done by streak plate method on M-9 medium (Anamika et al., 2013). Based on the production of pink zone around the colonies in secondary screening by agar well diffusion method, the excellent, good and fair l-asparaginase producers was detected.

Identification was performed based on Bergey's Manual of the Determinative Bacteriology (1975).

### ➢ Studies on Growth Parameters of Maximum L-Asparaginase Producers:-

Growth parameters like growth curve, effect of temperature, effect of pH were studied as per Kushwaha et al., 2012, so that the stage on which stationary phase is reached could be determined, and also to know that optimum parameter determined and used during fermentation procedure.

100 ml of nutrient broth was prepared and autoclaved. 1 ml of inoculums (24 hours old in nutrient broth) was added and incubated in shaker for 24 hrs. After that O.D. was read at 600 nm everyday till decline phase was not reached. Similarly the inoculated broth was incubated at 25°C, 37°C, 50°C, and 100°C respectively to study effect of temperature on growth and for the effect of pH each flask were maintained at pH 5, 7, 9 and 11 respectively. Each nutrient broth the flasks were incubated at 37°C for 24 hrs. After 24 hrs of incubation absorbance was read at 600 nm.

### ➢ Protein Estimation, Enzyme Assay and Optimization Studies of Crude Enzyme:-

Protein estimation was done by Lowry's method (1951) where a standard graph of Bovin Serum Albumin was prepared. The protein was estimated from crude enzyme from standard graph of BSA.

The L-asparaginase assay was determined by measuring released ammonia. One unit releases one micro mole of ammonia per minute at 37°C under the specified conditions. The standard graph of ammonium sulphate was plotted with the help of this graph l-asparaginase was assayed from crud enzyme by taking OD at 425 nm (Kushwaha et al., 2012).

Further the l-asparaginase was characterized to check effect of pH, temperature, metal ion, enzyme concentration and substrate concentration by maintaining respective pH, respective temperature, metal ions, substrate and enzyme concentration

(Kushwaha et al., 2012).

**RESULTS AND DISCUSSION :-**

➢ **Isolation, Identification and Screening of Maximum L-Asparaginase Producers:-**

Bacteria from soil were isolated by serial dilution method. Mixed cultures obtained were purified by the help of streaking on agar plate. Total 65 pure colonies were obtained which then screened for L-asparaginase positive activity.

All the 65 purified isolates were screened for L-asparaginase production and results of zone formed by the cultures were quantified based on the pink zone. Then the isolates were screened for primary and secondary screening.

Loopful of purified culture was streak on M -9 media, the presence of pink color around the colonies after 24 hrs at 370C incubation showed positive L-asparaginase activity. In primary screening out of 65 purified colonies 10 found to showed asparaginase positive. The secondary screening of bacterial isolate for L-asparaginase production was done on modified M-9 medium by agar well diffusion method and on the basis pink color zone, excellent, good or fair L-asparaginase producers were decided as given in Table No.1.

**Table No.1:-Screening for maximum L-asparaginase producers.**

| Sr.No. | Sites of Sample Collection | Isolates | Character |
|---|---|---|---|
| 1 | Patur | SP1 | +++ |
| 2 | Patur | SP2 | +++ |
| 3 | Gudadhi | SG3 | +++ |
| 4 | Kapshi | SK4 | +++ |
| 5 | Shirla | SS5 | + |
| 6 | Malkapur | SM6 | ++ |
| 7 | Deulgoan. | SD7 | + |
| 8 | Ridhora | SR8 | ++ |
| 9 | Ridhora | SR9 | +++ |
| 10 | Chinchkhed | SC10 | +++ |

**+++ = Excellent, ++ = Good, + = Fair.**

The isolate SP1, SP2, SG3, SK4, SR9, and SC10 showing maximum zone was selected for further studies and was identified by performing and comparing various morphological, cultural and biochemical tests according to the Bergey's Manual of Determinative Bacteriology (1975). Based on Bergey's Manual of the Determinative Bacteriology the isolates were identified as Bacillus sp, P. aeruginosa, Serratia sp, and S. aureus(Table 2).

**Table 2: Morphological and Biochemical Characteristics of Maximum L-asparaginase Producers.**

| Sample No. | | SP1 | SP2 | SG3 | SK4 | SS5 | SM6 | SD7 | SR8 | SR9 | SC10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sites of Sample Collection | | Patur | Patur | Gudhadi | Kapshi | Shirla | Malkapur | Deulgoan. | Ridhora | Ridhora | Chichkhed |
| Shape | | Circular | Circular | Circular | Circular | Circular | Circular | Circular | Circular | Circular | Circular |
| Margine | | Entire | Entire | Discrete | Entire | Entire | Entire | Entire | Discrete | Discrete | Entire |
| Elevation | | Raised | Raised | Flat | Convex | Flat | Convex | Raised | Flat | Flat | Flat |
| Colour | | White | White | Green | Yellow | Red | Yellow | White | Green | Green | Red |
| Surface | | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth | Smooth |
| Opacity | | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque | Opaque |
| Gram Character | | Gm + ve short rods | Gm + ve short rods | Gm - ve short rods | Gm +ve cocci in cluster | Gm – ve rods | Gm +ve cocci in cluster | Gm +ve rods | Gm - ve short rods | Gm - ve short rods | Gm – ve rods |
| I | | - | - | - | - | + | - | - | - | - | + |
| MR | | + | + | - | + | + | + | + | - | - | + |
| VP | | + | + | - | - | - | - | + | - | - | - |
| Citrate | | - | - | + | + | - | + | - | + | + | - |
| Glucose | A | + | + | + | + | + | + | + | + | + | + |
| | G | + | - | - | - | - | + | - | - | - | + |
| Lactose | A | + | + | - | + | + | + | + | - | - | + |
| | G | - | - | - | - | + | - | - | - | - | |
| Mannitol | A | + | + | - | + | + | + | + | - | - | + |
| | G | + | + | - | - | + | - | + | - | - | + |
| Amylase | | + | + | - | + | - | + | + | - | - | - |
| Catalase | | + | + | + | + | + | + | + | + | + | + |
| Oxidase | | + | + | + | + | + | + | + | + | + | + |
| Urease | | + | + | - | + | - | + | + | - | | |
| Probable Isolate | | Bacillus sp | Bacillus sp | P. aeruginosa | S. aureus. | Serratia sp. | S. aureus | Bacillus sp. | P. aeruginosa | P. aeruginosa | Serratia sp. |

➢ **Studies on Growth Parameters of Maximum L-Asparaginase Producers:-**

Growth curve plotted for incubation time in hrs against optical density. OD recorded after each 24 hrs. Maximum growth was observed after 72 hrs of incubation period, after that the growth curve was decline for all isolates.

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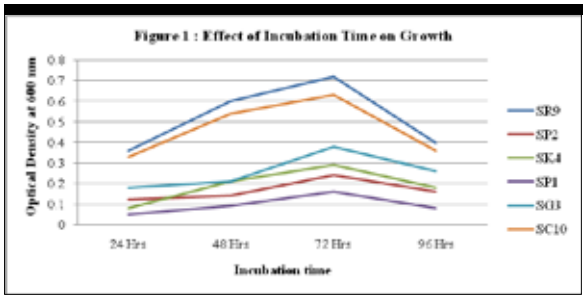
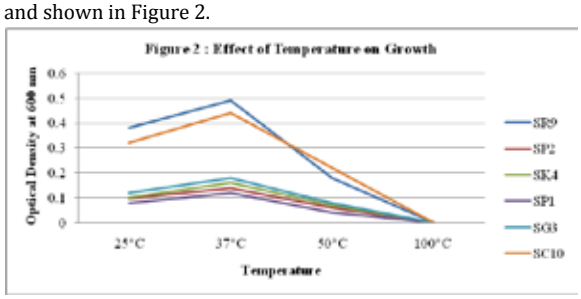
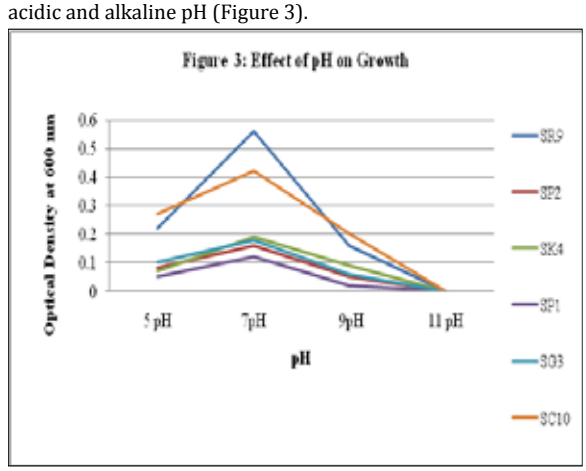



Effects of temperature on Growth of bacterial isolates were studied. All the isolates showed maximum growth at 37°C as temperature increases to 50°C less growth was observed. While at 100°C the growth was not observed. Also at low temp. ie. 25°C less growth as compare to optimum temperature was observed and shown in Figure 2.




The effect of different pH on the bacterial growth was studied. It was observed that at pH 7 maximum growth found than at acidic and alkaline pH (Figure 3).




➢ **Protein Estimation, Enzyme Assay and Optimization Studies of Crude Enzyme:-**

In order to find the concentration of protein in enzyme sample, a standard graph with known concentration of a standard protein (BSA) was plotted as shown in Table No.3.

**Table 3: Protein Estimation from crude enzyme**

| BSA (0.2mg/ml) (in ml) | Distilled water (in ml) | Conc. of BSA (mg/ml) | Reagent C (in ml) | Incubated at room temperature for 15 mins | Reagent D (in ml) | Incubated for 30 mins in dark and read OD at 660 nm. | Conc. for BSA | Conc. of Protein for crude enzyme |
|---|---|---|---|---|---|---|---|---|
| 0.0 | 1.0 | 0.0 | 5 | | 0.5 | | 0.0 | 0.99 |
| 0.2 | 0.8 | 0.4 | 5 | | 0.5 | | 0.21 | 0.95 |
| 0.4 | 0.6 | 0.8 | 5 | | 0.5 | | 0.39 | 0.95 |
| 0.6 | 0.4 | 1.2 | 5 | | 0.5 | | 0.46 | 0.94 |
| 0.8 | 0.2 | 1.6 | 5 | | 0.5 | | 0.80 | 0.85 |
| 1.0 | 0.0 | 2.0 | 5 | | 0.5 | | 0.99 | 0.82 |

Protein estimated from the crude enzyme from cell free supernatant of isolates by Folin Lowery method. Isolate no. SR9 found to have highest protein content of 0.99 mg per ml. It was followed by SP2 and SK4 in which 0.95 mg / ml protein found. While crude enzyme from SG3, SP1 SC10 by contain 0.94, 0.85 and 0.82 mg/ ml respectively.

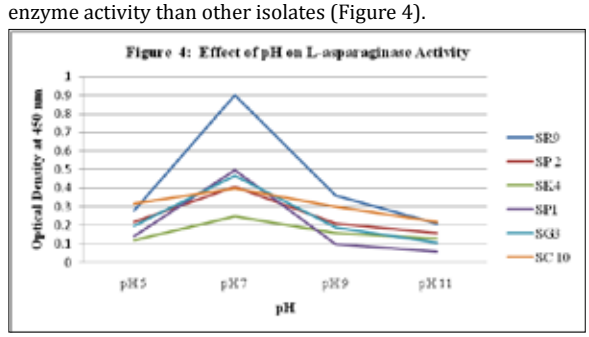
Amount of ammonia release from the crude sample of isolates can be calculated by plotting standard graph ammonium sulphate in order to assay the activity of L-asparaginase enzyme. The standard graph of ammonium sulphate was plotted. From the standard graph it was calculated that SR9 (P. aeruginosa) showed 0.61 /mg activity which was highest to all. This was followed by isolate SP2 (Bacillus sp.), SK4 (S. aureus), SG 3 (P. aeruginosa), SP1 (Bacillus sp.), and SC 10 (Serratia sp.) which showed 0.47 /mg, 0.36 /mg, 0.21/mg, 0.19 /mg and 0.13/mg activity respectively (Table 4).

**Table 4: L-Asparaginase Assay in Crude Enzyme**

| Sr. No. | Isolate No. | OD at 425 nm | Activity (U/Mg) |
|---|---|---|---|
| 1 | SR9 | 0.96 | 0.61 |
| 2 | SP2 | 0.31 | 0.19 |
| 3 | SK4 | 0.74 | 0.47 |
| 4 | SG3 | 0.34 | 0.21 |
| 5 | SP1 | 0.21 | 0.13 |
| 6 | SC10 | 0.57 | 0.36 |

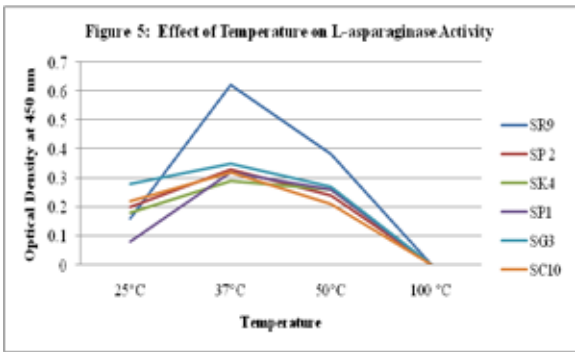
The crude enzyme was also characterized for the effect of pH, temperature, metal ions, enzyme concentration and substrate concentration and results was noted as follows.

Effect of pH on L-asparaginase activity was studied which showed that at neutral pH 7 maximum enzyme activity obtained. At acidic pH 5 enzyme activity was low and at alkaline pH 9 and pH 11 less activity was observed as compared to pH 7. The enzyme from Isolate No SR9 was found to have highest enzyme activity than other isolates (Figure 4).

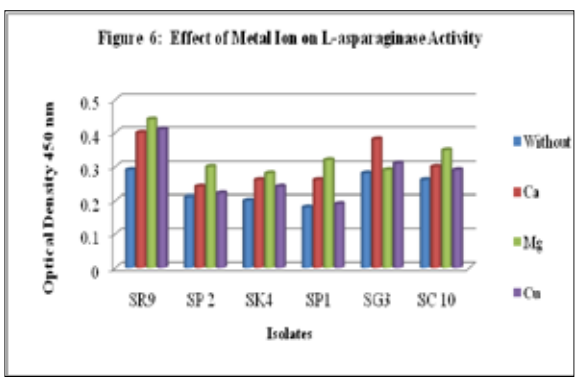



Effect of temperature on L-asparaginase activity was studied (Figure 5). It was observed that at 37°C maximum enzyme activity was observed for enzymes from all isolates. At low temperature enzyme activities were lowered and at higher temperature it shows less activity as compared to 37°C. When there was again increase in the temperature up to 100°C enzyme activity was found zero. Isolate No SR9 was found to exhibit highest en-

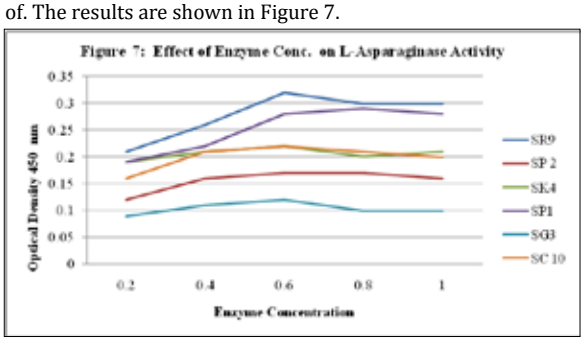
zyme activity than other isolates at 37°C which is about 0.39 µ/ mg.




During the study of effect of different metal ion on L-asparaginase activity was observed that calcium, magnesium and copper enhance the enzyme activity, but magnesium was found good activator as compared to other except for crude enzyme from isolate no. SG3 for which it as has calcium as good activator. The results were noted and shown in Figure 6.




In the enzyme concentration study was observed that as increasing the concentration of enzyme the enzyme activity also increases up to certain limits but after that enzyme activity level of. The results are shown in Figure 7.




Similarly increasing the concentration of substrate the enzyme activity also increases up to certain limits but after that enzyme activity does not alter. The activity of crude enzyme from isolates at various substrate concentrations is given in Figure 8.

**CONCLUSION:**

The farm soil found to be good source for the isolation of L-asparaginase producers as 6 bacterial isolates were screened as maximum L-asparaginase producers. The L-asparaginase producing isolates were found to belong to genera Bacillus, Pseudomonas, Staphylococcus and Serratia. The isolate SR9 (P. aeruginosa) showed highest L-asparaginase activity than others. Thus the isolate SR9 (P. aeruginosa) and other also can be good sources for the production of L-asparaginase on large scale. The enzyme production can be optimized to further by studying various parameters like effect of time course, effect of various carbon and nitrogen sources which can be also helpful for reducing the cost of production. Further studies on purification and protein structure prediction of L-asparaginase from these bacterial isolates can contribute to the therapeutic value of these enzymes.

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