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Research Article

SCREENING AND CHARACTERIZATION OF L-ASPARAGINASE PRODUCING BACTERIA FROM SOIL SAMPLE

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Abstract- Asparaginase isolated from muddy soil, collected from cancer hospital garden and some other places of Gwalior. L-Asparaginases have been used as an anti-tumor agent for the effective treatment of acute lymphoblastic leukemia and food processing and reduce the acrylamide formation during frying of starchy food at high temperature. In the present study, we isolate bacteria (producing asparaginase enzyme) strains from soil. The isolated bacteria were screened for L-Asparaginase producing using M-9 medium on the basis of pink zone formation. An enzyme isolated from the bacterium *Escherichia coli* or *Erwinia carotovora* with anti-leukemic activity. L-Asparaginase are hydrolyzed into L-aspartic acid and ammonia in leukemic cells, resulting in the depletion of asparagine, inhibition of protein synthesis, cell cycle arrest in the G1 phase, and apoptosis in susceptible leukemic cell populations. The *Erwinia carotovora* -derived form of asparaginase is typically reserved for case. At present, the principal source of L-asparaginase for clinical trials is the bacteria *E. coli*; several other alternative sources are screen for production of large quantities of L-asparaginase then *E. coli*.

Keywords- *De- novo, Erwinia carotovora, L-Asparaginase, Lymphoblastic leukemia*

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Introduction

L-Asparaginase is an effective anti-leukemic agent in mice and rats. Currents clinical studies indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell disease in man. At present, the principal source of L-asparaginase for clinical trials is the bacteria *E. coli*; several other alternative sources are screen for production of large quantities of L-asparaginase then *E. coli*. L-asparaginase an enzyme isolated from the bacterium *Escherichia coli* or the *Erwinia carotovora* with anti-leukemic activity. Asparaginase are hydrolyzes in to L-aspartic acid and ammonia in leukemic cells, resulting in the depletion of asparagine, inhibition of protein synthesis, cell cycle arrest in the G1 phase, and apoptosis in susceptible leukemic cell populations. The *E. carotovora* derived form of asparaginase is typically reserved for case. Several micro-organisms including Serratia marcescens produce L-asparaginases with antitumor activity. L-Asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. The precise mechanism of its action is still unknown although hydrolysis proceeds in two steps via a beta-acyl-enzyme intermediate. Asparaginase enzyme is produced throughout the world by submerged fermentation in soil bacteria. Solid-state fermentation is a very effective technique as the yield of the product is many times higher. When compared to that in, and it also offers many other advantages. Microbial asparaginases have been particularly studied for their applications as therapeutic agents in the treatment of certain types of human cells. Asparagine can either be produced within a cell through an enzyme called "asparagine synthetase" or it can absorb into the cell from the outside. Tumor cells, more specifically lymphatic tumor cells, require huge amounts of asparagine's to keep up with their rapid, malignant growth. This means they use both asparagine from the diet as well as what they can make themselves to satisfy their large asparagine demand. Broome in 1961 [1] discovered that the regression of lympho-sarcoma transplants in mice treated with guinea-pig serum was due to the nutritional dependence of the malignant cells on exogenous L-asparagine.

Curran *et al* [2] first reported deamidation of L-asparagine by extracts of *E. coli* in commercial production of L-asparaginase appeared desirable only after Mashburn and Wriston [3] showed that L-asparaginase from *E. coli* inhibits tumors in mice.

Materials and Methods

Sample Collection

The soil samples were collected from different places of Gwalior regions. To 3 cm sediment sample was taken out with the help of a sterile spatula and this sample was transferred to a sterile polythene bags and transport to laboratory. The samples were air dried at room temperature.

Isolation of bacteria from the soil sediments

1gm of soil was dissolved in 100 ml of distilled water and 1ml of serial dilution was spread plated on nutrient agar medium using dilution plate's technique. All the plates were incubating at 30°C in an incubator for 24 hours. After incubation colonies were selected and Streak it on modified M-9 medium.

Screening for L-Asparagine Production

The bacterial strains were grown on M-9 media for 24 hours for identification of asparaginase producing bacteria. The modified M-9 mediums incorporate with phenol red as pH indicator will be used. L-asparaginase activity of bacteria was identified by formation of pink zone around colonies after overnight incubation of culture.

Characterization of bacteria isolate

Isolated colonies were identified, identification of isolated colonies was carried out by simple staining, grams staining and motility testing by hanging drop method. Biochemical characterization was used for characterization of bacteria.

Morphological Characterization
The shape of the bacteria was determined by light microscopy after gram staining, motility test was examined by test on motility agar and spores are observed by spore staining.

Gram Staining
Both Gram+ve and Gram–ve organism from a complex of crystal violet iodine within the bacterial cell during the gram staining procedure. Gram+ve organisms are through to resist decolorized by alcohol and acetone because cell wall permeability is markedly decrease when it is dehydrated by this solvent. Thus, the dye complex is entrapped within the cell, resist being washed out by the solvent, and Gram+ve bacteria remain purple following this differential stain. In contrast cellwall permeability of Gram-ve organisms is increased by ethyl-alcohol washing because it removes the outer membrane from the Gram-ve cell wall. These allow removing of crystal violet –iodine from within the cell. The decolorized Gram-ve bacteria cell can be rendered visible with a suitable counterstain, in this case Safranin, which stained them pink. Pink which adhered to the Gram+ve bacteria is marked by the purple of the crystal violet.

Spore Staining
Spore have a durable outer coating that is composed of the protein keratin. This keratin coat resists staining. So, it in order to stain a spore the primary stain, malachite green must be heated to drive the stain into the spore. Vegetative cells are then decolorized with water and 0.5% Safranin is used to counter stained. Thus, endospore was stained green, while vegetative cells are stained red.

Motility Test
The media contains small amount of agar and gelatin as well as triphenyltetrazolium chloride (TTC). TTC is a soluble compound that is taken up by the bacterial cell. Once the bacteria have absorbed the substance, it is reduced releasing the acid formation a highly pigmented red. Insoluble compound motility is indicated by the lateral diffusion of colour throughout the media.

Biochemical Characterization
For biochemical tests the culture was grown on nutrient agar and tests were performed as described by Lauinger *et al* [4] and Kristiansen *et al* [5].

Catalase Tests
24 hours old culture was placed on glass slide and one drop of hydrogen peroxide was added to it. The formation of bubbles indicates the catalase production.

Gelatin Test
The agar contained basal medium with 1% gelatin was prepared and inoculated the isolate. The culture incubates at 37°C for 24 hours and hydrolysis was absorbed.

Mannitol Salt Agar
Staphylococci ferment mannitol and form yellow mannitol enzymatic digest of casein, enzymatic digest id the carbohydrates source. In high concentration of sodium chloride inhibits most bacteria other than the Staphylococci. Phenol red is the pH indicator and beef extracts provided the nitrogen, vitamins and carbon in mannitol salt agar

Sugar Fermentation Test
The principal of sugar fermentation is that it results from incomplete breakdown to glucose molecule, when glucose is breakdown to pyruvic acid in the presence of anaerobic respiration.

Indole acid Test
This test is done to determine if bacteria can break down to amino acid tryptophan into indole acid. SIM media (tryptic soy broth) is inoculating using a transfer needle.

Citrate Utilization
Test for the ability of bacteria to convert citrate (an intermediate of citric acid cycle). In this media citrate is not only carbon source available to the bacteria. If it cannot use citrate then it will not grow. If it can use citrate then it will grow and media will turn a bright blue as a result of an increase in pH of the media. To inoculate this slant uses the transfer loop.

MRVP (Methyl Red- Voges–Proskauer)
This test is use to determine two things; the MR portion is used to determine that if glucose can be converted to acetone. The tests are performed by inoculating a single tube of MRVP media with help of transfer loop and then allow the culture to grow for 3-5 days. After the growing of culture about half of the culture is transferred to a clean tube. One tube is use to conduct test, the second tube is serve as VP test.

MR (Methyl Red test)
Methyl red is added to MR tube. A red colour indicate a positive test (glucose can be converted into the acidic end products such as lactate and acetate. A yellow colour indicate a negative result, glucose is converted into neutral end products.

VP (Voges Proskauer test)
First alpha naphthol (Barritt's reagent) and then potassium hydroxide (also called Barritt's reagent B) are added to VP tube. The culture is allowed to it for about 15 min. For colour development occurs. If acetone is produced then colour turn red colour (positive result). If acetone was not produced then the colour appear yellowish to copper in colour (a negative result).

Results and Discussion
Morphological Observation
The collected bacterial sample is shown morphology in table 1. The morphology of bacteria is found in different shape like, bacillus, cylindrical, rod, coccus, short rod, short bacillus and long rod in different sample. Sample T-12B, T-11C, T-13D, T-30, and T-15A were found Gram (-) and bacilli form and sample T-29 was found Gram (-) and Terminal and Cylindrical forms, sample T-13A, T-15C were found Gram (-) and rod form, S-12C, S-16D were found Gram (+), short bacilli and rod form respectively, S-19C and S-15E were found Gram (-) and short rod form. The sample Z-11D, Z-30D, Z-29C and Z-15A were found Gram (+) and rod, short rod, cocci and rod respectively. Sample S-16G was found Gram (-) and short rod form. Sample S-18A and S-17C were found Gram (+) and short rod form. Sample S-17A and S-17F S-11B were found Gram (-) and long rod and rod form respectively. The bacterial sample S-11B were found Gram (+) and short rod form. The result reveals that mostly bacteria were found Gram (-) and rod shaped.

Table-1 *Morphological observation of the samples*

SN	Sample Name	Results	Morphology
1	T-12 B	Gram (–)	Bacilli
2	T-11 C	Gram (–)	Bacilli
3	T-13 D	Gram (–)	Bacilli
4	T-30 B	Gram (–)	Bacilli
5	T-15 A	Gram (–)	Bacilli
6	T-29 D	Gram (–)	Terminal and Cylindrical Spores
7	T-13 A	Gram (–)	Rod
8	T-15 C	Gram (–)	Rod
9	S-12 C	Gram (+)	Short Bacilli
10	S-16 D	Gram (+)	Rod
11	S-19 C	Gram (–)	Short Rod
12	S-15 E	Gram (–)	Short Rod
13	Z-11 D	Gram (+)	Rod
14	Z-30 D	Gram (+)	Short Rod
15	Z-29 C	Gram (+)	Cocci
16	Z-15 A	Gram (+)	Rod
17	S-16 G	Gram (–)	Short Rod
18	S-18 A	Gram (+)	Short Rod
19	S-17 C	Gram (+)	Short Rod
20	S-17 A	Gram (–)	Rod
21	S-17 F	Gram (–)	Long Rod
22	S-11 B	Gram (+)	Short Rod

Table-2 *Biochemical characterization of Gram-positive bacteria*

SN	Tests	S-12 C	S-16 D	Z-11 D	Z-30 D	Z-29 C	Z-15 A	S-18 A	S-17 C	S-11 B
1	Spore Staining	No	Terminal	Terminal	Central	+ve	+ve	+ve	+ve	+ve
2	Catalase Test	+VE	+VE	+VE	+VE	Central	No	No	Terminal	Terminal
3	Msa	NO	-VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE
4	Motility Test	Non motile	Motile	Motile	Motile	+VE	NO	NO	+VE	+VE
5	Gelatin Test	+ve	+ve	+ve	+ve	Non motile	Motile	Motile	Motile	Motile
Sugar Fermentation Test										
6	Glucose	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	NO Fermentation	Complete fermentation	Complete fermentation
7	Fructose	No fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation
8	Sucrose	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	No fermentation	No fermentation	Complete fermentation
9	Maltose	Complete fermentation	No fermentation	Complete fermentation	Complete fermentation	Partial fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation.
10	Mannitol	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	No fermentation	Complete fermentation	Complete fermentation
11	Bacteria Name	*Planococcus*	*Bacillus*	*Bacillus*	*Sporosarcina*	*Sulfodobacillus*	*Marinococcus*	*Kurthia*	*Oscillospira*	*Bacillus*

Table-3 *Biochemical Tests for Gram Negative Bacteria*

SN	IMVIC TEST	T15-A	T13-A	S12-C	S16-D	S15-E	S 17-F	S19-C	S13-D	S17-A	S16-G
1	Indole	+VE	+VE	+VE	+VE	+VE	+VE	+VEE	+VE	+VE	+VE
2	MR Test	-VE	+VE	+VE	+VE	-VE	-VE	-VE	-VE	-VE	-VE
3	VP Test	-VE	-VE	-VE	-VE	-VE	-VE	-VE	-VE	-VE	+VE
4	Citrate Test	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE	+VE
5	Motility Test	Motile	Motile	Non motile	Motile	Motile	Motile	Motile	Motile	Non motile	Motile
Sugar Fermentation Test											
6	Glucose	No fermentation	Complete fermentation	Partial fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	No fermentation	Partial fermentation	Complete fermentation
7	Fructose	Complete fermentation	Complete fermentation	Partial fermentation	No fermentation	Complete fermentation	Complete fermentation	Partial fermentation	Complete fermentation	Complete fermentation	Complete fermentation
8	Sucrose	Partial fermentation	No fermentation	Complete fermentation	Complete fermentation	Complete fermentation	No fermentation	Complete fermentation	Partial fermentation	Partial fermentation	Complete fermentation
9	Maltose	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Partial fermentation	No fermentation	Complete fermentation	Complete fermentation	Partial fermentation
10	Mannitol	Complete fermentation	No fermentation	Complete fermentation	Partial fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	Complete fermentation	No fermentation
11	Name of bacteria	*Kluyvera ascorbata*	*Serratia odorifera biogroup2*	*Edwardsiella tarda*	*Kluyvera ascorbata*	*Erwinia uredovora*	*Xenorhabdus luminescens*	*Erwinia chrysanthemi*	*Xenorhabdus nematiphilus*	*Erwinia ananas*	*Erwinia uredovora*

Conclusion

Total 40 samples were collected from different regions and sample proceeds on modified M-9 media for screening of L-Asparaginase producing bacteria. There were screened nine bacteria as a positive culture on the basis of pink zone on modified M-9 media, which indicates positive asparagine. Isolates were screened and carried out for the characterization through different biochemical tests. 13 bacteria are gram negative, remaining 09 are gram positive. On the basis of biochemical test, I identified the bacteria which produce L-Asparaginase enzyme. Identified bacteria are 10 *Bacillus*, 5 *Sporosarcina*, 2 *Planococcus* and 1 Kurthia.

Application of research: Study of L-asparaginase producing bacteria from soil sample.

Research Category: Medical microbiology

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