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Production Of L-Asparaginase From Soil Isolates At Different P^{H} And Temperature

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ABSTRACT

Asparaginase is an enzyme and it is used as chemotheraptic agent for a treatment of human cancer and acts as a catalyst in the breakdown of asparagines to aspartic acid and ammonia. Asparagine is a nutritional requirement of both somatic and cancer cells.

The L-asparaginase is an anti neoplastic agent, used in the lymphoblastic leukemia, Synthesis L-asparagine due to the absence of L-asparagine synthase. For this reason the commonest therapeutic practice is to inject interavenously free enzyme in order to decreases the blood concentration of L-asparagine affecting selectively the neo plastic cells. How ever L-asparaginase from bacterial origin can cause hypersensitivity in the long term used leading to allergic reaction and anaphylaxis. The search for L-asparaginase sources like eukaryotic microorganisms can lead to an enzyme with less adverse effect. The importance of microorganisms as L-asparaginase sources has been focused seens the time, it was obtained from E.coli and its anti-neoplastic activity demonstrated in guniapig serum.

Reseacher need to study about Production of L-asparaginase from soil isolates at different pH and temperature .

KEY WORDS: L-asparaginase, soil isolates, Erwinia enzyme

INTRODUCTION

The E. coli, Erwinia enzyme were isolated and purified and experimentally used as an anti leukemia agent in human patients. It demonstrated high potential against children's acute lymphoblast leukemia. Several recharch groups have studied asparaginase production and purification in attempt to minimize impurities the produced allergic reaction (Campbell et.al 1967, Boss 1997, Gallagher et.al 1999).

Major sources of enzyme are living organisms like plants, animals and microorganisms. Of the sources microbial enzyme accounts for the major value for applications in industries and medicine. There are two types of enzymes endoenzyme and exoenzyme. The enzyme produced by a cell, function within that cell and hence are called endoenzyme. Some enzymes liberated by living cells and catalyzed reactions in the cell environment such enzyme are known as exoenzyme. Micro orgasms are able to produce both extracellular and entracellular enzymes.

Asparaginase is an enzyme and it is used as chemotheraptic agent for a treatment of human cancer and acts as a catalyst in the breakdown of asparagines to aspartic acid and ammonia. Asparagine is a nutritional requirement of both somatic and cancer cells. A low level of nonessential amino acid, asparagine only affect the viability of the normal cells have an asparagine synthetase for the synthesis of asparagine from aspartic acid where cells have low level of this enzyme. Asparagine is produced by variety of microbial sources including fungi, yeast and bacteria. Asparagines from E.coli and Erwinia species have been used as antitumar and anti-leukemia agent. The utilization of asparaginase from the above mentioned sources was initially limited because of its potential toxicity and several side effects. Endophytic fungi from Thai medicinal plant were evaluated for their ability to produce L- asparaginase.

L-asparagines (E.C. 3.5.1.1) are enzymes that catalyze the hydrolysis of L-asparagine to L-aspartate and ammonia. Using amino acid sequences and biochemical properties as criteria, enzymes with asparagines activity can be divided into several families (Borek 2001). The two largest and best characterized families include bacterial and plant-type asparaginases. The L-asparaginases is tetrameric With high molecular weight of about 1,41,000 daltons. The bacterial-type enzymes have been studied for over 40 years (Campbell ET al.1967), mostly because they are important agents in the therapy of certain types of lymphoblastic leukemias (Gallagher et al. 1989). Their homologues are found in some mammals and in fungi (Bonthron and Jaskolski 1997). The bacterial-type enzymes frequently exhibit other activities as well, and this family may be significantly larger than the collection of sequences deposited as asparaginases. In particular, enzymes such as glutamin-(asparagin)-ases (EC 3.5.1.38) (Ortlund et al. 2000), lysophospholipases (EC 3.1.1.5) (Sugimoto et al. 1998) and the α-subunit of Glu-tRNA amidotransferase (EC 6.3.5.-) (Tumbula et al. 2000), can also be considered part of the bacterial asparagines family.

The L-asparaginase is an anti neoplastic agent, used in the lymphoblastic leukemia, Synthesis L-asparagine due to the absence of L-asparagine synthase. For this reason the commonest therapeutic practice is to inject interavenously free enzyme in order to decreases the blood concentration of L-asparagine affecting selectively the neo plastic cells. How ever L-asparaginase from bacterial origin can cause hypersensitivity in the long term used leading to allergic reaction and anaphylaxis. The search for L-asparaginase sources like eukaryotic microorganisms can lead to an enzyme with less

adverse effect. The importance of microorganisms as L-asparaginase sources has been focused seens the time, it was obtained from E.coli and its anti-neoplastic activity demonstrated in guniapig serum.

Chemical data of L-asparaginase -

Formula- $C_{1377}H_{2208}N_{382}O_{442}S_{17}$

Mol. Mass- 31731.9 g/mol.

L-Asparagine **-**

L-asparagine is one of the twenty most common natural amino acids on earth. It has carboxamide as the side chain's functional group. It is considered non-essential amino acid. Its codons are AAU and AAC.L-asparagine was first isolated in 1806 form asparagus juice, in which it is abundant hence its name becoming the first amino acid to be isolated. It play role as "capping" the hydrogen bond interactions which would otherwise need to be satisfied by the polypeptide backbone.

SOURCES-

Dietary sources- Asparagine is not an essential amino acid, which means that it can be synthesized from central metabolic pathway intermediates in humans and is not required in the diet. Asparagine is found in:

Animal sources: dairy, beef, poultry, eggs, fish, lactalbumin, seafood.

Vegetarian sources: Asparagus, potatoes, legumes, nuts, seeds, soy, whey, whole grains.

Degradation-

Aspartate is a **glucogenic** amino acid. **L-asparginase** hydrolyzes the amide group to form aspartate and ammonium. A transaminase converts the aspartate to oxaloacetate which can then be metabolized in the **citric acid cycle** or **gluconeogenesis**.

Function

The nervous system needs asparagine to maintain the equilibrium, as well as in amino acid transformation. It also plays an important role in the synthesis of ammonia.

L-asparaginase is an enzyme that destroys asparagine external to the cell. Normal cells are able to make all the asparagine they made internally whereas tumor cells become depleted rapidly and die.

Sources of L-asparaginase enzyme- This enzyme is present in many animal tissues, bacteria, plants and in the serum of certain rodents but not in man. *Pisum sativum*_one of the plant source of L-asparaginase enzyme in case of animal it is mainly obtain from

Guinea pig serum. As far as microorganisms as a source of enzyme concerned bacteria, fungi and actinomycetes are good source of L-asparaginase enzyme. Following are the examples of L-asparaginase enzyme producers.

It is used as chemotherapeutic agent for the treatment of various types of cancers.It is used for seed viability and germination.For N_2 mineralization (Blagoveshchenskaya and Danchenkos, 1974), this was first detected in soil by Drobnik (1956). (Text book of biotechnology of biofertilizer).L-asparaginase plays an important role in amino acid metabolism and mineralization of N_2 and C compound in aquatic environment. By considering these applications of L-asparaginase enzyme we decided to work on "Screening of high yielding microorganisms and to obtain L-asparaginase enzyme from them".

The present research study is focused on the standard procedure for the production of L-asparaginase from different soil isolates and characterizations of produced L-asparaginase.

OBJECTIVE OF THE STUDY:

- To study the Production of L-asparaginase from soil isolates at different P^H and temperature.

PHASES OF THE STUDY :

The present research work is divided into following phases.

- Collection of soil sample.
- Isolation of soil isolates
- Enrichement of soil isolates.
- Production of L-asparaginase

Optimization of enzyme with reference to different parameter will be studied as follows,

- Substrate concentration.
- P^H
- Temperature.

REVIEW OF LITERATURE

The enzyme L- asparaginase is tetra meric with high molecular weight of about 1,41,000 daltons. L- asparaginase produced by various strains of E.coli were active in elaborating L- asparaginase. Also Erwinia carotovora produce L- asparaginase which is serologically and biochemically distinct from asparaginase of E.coli. Although both these asparaginase useful in treatment of some form of cancer with same anti-neoplastic activity and with same adverse effect.

(Vishal P. Oza, Shraddha D.) The plant withania somnifera was identified as potential sorce of enzyme L- asparaginase which have highly specific activity. Purification was carried out by combination of protein precipitation with ammonium sulphate as well as sephadex-gel filtration. The purified enzyme is homodimer, with a molecular mass of 72± 0.5kDa as estimated by SDS-PAGE. The enzyme has pH optimum of 8.5 and an optimum temperature of 37^0c. The Km value for the enzyme is 6.1 x 10. It has isoelectric point at P^H 4.9.

From bacterial culture it is advantageous to start purification of enzyme from highly active cell, culture condition necessary for good growth and high enzyme yield were studied. Gentle aeration proved suitable for good growth as well as high enzyme content.

Tobias 0. Yellint and John C. Wriston. Jr**.** (1966) reveald that a sevenfold purification of guinea pig serum asparaginase was reported some time ago (Meister, 1955). Interest in this enzyme has increased recently, however, both because of its use in assaying for L-asparagine (Tower *et al.,* 1963) and because the enzyme is the agent in GPS responsible for the antilymphoma activity of the serum, as first suggested by Broome (1961).They reported here the isolation of a 900-fold purified Guinea pig serum L-asparaginase which is homogeneous by several criteria, including sedimentation equilibrium ultracentrifugation and immunoelectrophoresis, and to describe certain properties of this enzyme. The molecular weight was found to be *ca.* 138,000 by equilibrium sedimentation, and 133,000 based upon elution volume from G-200 Sephadex. They determined *protein* concentration according to Waddell (1956) and Asparaginase activity was determined by direct nesslerization method.

Howard Cedar and James H. Schwartz (1968) studied some of the condition which controls the production of asparaginase II of Escherichia *coli.* They showed that L-asparaginase II was synthesized at constant rates by *E.Coli* under anaerobic condition. They carried out L-asparaginase II enzyme production in standing culture. They obtained little enzyme from the cell during aerobic growth, that is they found that O_2 in inhibitory for enzyme production. They were concluded that anaerobiosis depressed the formation of L-asparaginase II. They also observed that little L-asparaginase II was formed either aerobically or anaerobically when the asparagine was the sole source of N_2.They found optimum P^H between 7 and 8 at 37^0C. for enzyme production. They also found that enzyme was produced at lower rates in presence of sugars: glucose was the most inhibitory.

R.E. Peterson and A. Ciegler (June 1969) surveyed the 123 species of bacteria for L-asparaginase synthesis, out of that Erwinia aroideae NRRL B-138 showed highest yields. They found that serological tests with antibodies sensitive to the L-asparaginase from E.Coli and S. marcescens proved the L-asparaginase from E. aroideae NRRL B – 138 to be immunological distinct.

M.H. Bilimoria (Dec1969) evaluates different *E.Coli* cultures with respect to their ability to produce the two L-asparaginases under different growth conditions. They showed that out of twenty-eight coliforms, five strains of *E.Coli* were particularly active in elaborating L-asparaginase 2, the form of the enzyme useful in the treatment of some forms of cancer. They studied that to start purification of the enzyme from highly active cells cultural condition necessary for good growth and high enzyme yield. L-asparaginase-2 has been purified about 40-fold by a combination of ammonium sulfate and ethyl alcohol precipitation.

R.E. Peterson and A. Ciegler (July1969) were obtained maximum yields of 1,250 IU (international unit)/g (dry weight of cells) of L-Asparaginase in 8hrs from *Erwinia aroidia* NRRL B-138.Partial purification and concentration of the extracted L-asparaginase yielded a preparation with an activity of 275 IU/ml. They also found that enzyme exhibited a pH optimum of 7.5 and a Km of 3 x 10^{-8} M. They found maximum activity between P^H 7 and 8. Enzyme activity was essentially absent bellow P^H 4.5. Varying substrate concentration gave them a Km of 3.0 x 10-3, indicating good affinity of the enzyme for the substrate concentration.

H.A. Campbell and L. T**.** Mashburn (1969) Shows that L-asparaginase EC-2 from *E.Coli* hydrolyzes L-glutamine and D-asaparagine but at a much slower rate than L-asparaginase, these amide activities were not separated by several different methods of enzyme purification .Also suggest that the hydrolysis occurs at the same active site of the protein. Inhibition of L-asparagine hydrolysis occurred with ammonia at pH value of 8.5. Now that *Escherichia coli* L-asparaginase EC-2 [1] has been demonstrated to be an active agent for the treatment of some tumors in man (Hill *et al.,* 1967; Oettgen *et al.,* 1967), the elucidation of its several enzymatic activities is of considerable importance. The low rate of L-glutamine hydrolysis by the *E. coli* L-asparaginase EC-2 has been reported (Campbell *et al.,* 1967) and shown to be a characteristic of this enzyme. In contrast, the L-asparaginase from guinea pig serum does not catalyze the hydrolysis of L-glutamine (Meister *et al.,* 1955). L-Glutamine is required for several metabolic pathways including the formation of L-asparagine by the enzyme L-asparagine synthetase (Patterson and Orr, 1967, 1968; Horowitz *et al.,* 1968; Prager and Bachynsky, 1968). Thus the L-glutaminase activity of EC-2 may be favorable for L-asparagine depletion during treatment.

MATERIALS AND METHODS

Materials :

1. **Special medium for detection of L-asparaginase producers –**

   Composition –

Yeast extract	-	0.3 g
L-asparagine	-	0.1 g
$NaHPO_4$	-	0.1 g
Nacl	-	0.5 g
Agar Agar	-	2.5 g
Distill water	-	100 ml
P^H	-	7.5

Preparation of medium –

All components were added to the distilled water and volume was made to 100 ml the medium was sterilized by autoclaving at 121 c for 15-18 minutes.

2. Modified M-9 agar

Composition:-

Na_2HPO_4. $2H_20$	-	6.0g
KH_2PO_4	-	3.0g
Nacl	-	0.5g
L-asparagin	-	5.0g
1 mole/1$MgSO_4.7H_2O$	-	2.0ml
0.2mole/1$Cacl_2.2H_2O$	-	1.0ml
20% glucose stock	-	10.0ml
Agar Agar	-	20.0gm
Distilled water	-	1000ml
P^H	-	7.0ml
Phenol red indicator	-	0.1 to 2.5 ml

Preparation:-

All component were dissolved in 1000ml Distilled water was sterilized at 121^0c.

For 15-20 minutes.

3. Modified M-9 broth (P^H 7.0)

All ingredients except agar agar of modified M-9 agar medium were added to Distilled water and volume made to 1000 ml.

Reagent:-

Nessler's reagent –

KI	-	50.0 gm
Ammonia free Distilled water	-	35.0 ml
$HgCl_2$ Solution (Saturated)	-	35.0 ml
KOH(50% aqueous solution)	-	400.0 ml
Distilled water	-	530.0 ml

METHODS :

1. **Collection Of Soil samples:-**

Soil from different sources (Garden soil and Farm soil) is used as source of L- asparaginase producing organisms. These samples were collected from Latur region.

2. **Enrichments for L-asparaginase producing microorganism :**

1gm of soil sample from both sources is added in previously saline tubes each containing 10ml saline. Then 1ml of suspension is added to sterile nutrient broth containing L- asparagine for enrichment at 37^0c.for 24 hours.

3. **Detection and screening of L- asparaginase producing microorganisms :**

After enrichment a loop full of the enriched medium is taken and stricked on selective agar medium containing M-9 L- asparagine as a source of nitrogen and disodium hydrogen phosphate (Na_2Hpo_4) as a source of phosphorus. All these plates were incubated at 37^0c. for 24 hours.

After incubation isolated colonies were picked up and inoculated in M-9 broth. Incubate these flask incubated on shaking incubated (110 rpm) at 37^0c. for 24 hr. A loopful suspension from the above enriched media was stricked on selective M-9 agar medium containing few drops of 2.5 % phenol red indicator. These plates were incubated at 37^0c. for 24 hr. after incubation the L-asparaginase producing organism were identified from the pink colored zone around the colonies. The colonies having maximum diameter of pink color zone were selected for further procedure.

4. **Production of L- asparaginase:-**

100 ml of modified M-9 broth was taken in 250 ml. flask and 0.5ml of the above inoculum of selected isolates from different soil sample. Proceed it for the production of L- asparaginase with different conditions like pH and temperature and incubate it for production.Repeat the same procedure for the remaining sample and put in for further production.

5. **Detection of L-asparaginase activity-**

Method used- Nesslerization method.

Material used- Optical density compare to standard graph of ammonia sulphates.

One international unit of L-asparginase is that amount of enzyme which liberates 1micromole of ammonia in 1 min at 37^0 C.

DATA ANALYSIS :

Table No. 1 :For determination of released ammonia, graph using ammonia standard was plotted.

Standard Protocol for Ammonia Calibration

Ammonium Sulfate Solution (ml)	Distilled Water (ml)	Nesslers Reagent (ml)	Optical density at 480 nm
	7.5	1.0	0.745
0.1	7.4	1.0	0.700
0.2	7.3	1.0	0.740
0.3	7.2	1.0	0.750
0.4	7.1	1.0	0.850
0.5	7.0	1.0	0.870
0.6	6.9	1.0	0.850
0.7	6.8	1.0	0.930
0.8	6.7	1.0	0.960
0.9	6.6	1.0	0.950
1.0	-	-	0.985

(Source: ……)

Keep the tubes at 37^0c for 10 min and take optical density at 480 nm. Plot the graph of (O.D.) optical density verses ammonia released.

Table No 2 : Garden Soil (Temp. 37^0c and P^H 7.0)

Centrifugation and take supernatant

Sr.No.	Particulars	ml	ml
1	Supernatant	0.5	0.5
2	Distilled Water	7.0	7.0
3	Nesslers reagent	1.0	1.0
	Incubate at 37^0c for 10 min.		
4	Optical density	0.900	-

(Source:……..)

Table No.3: Garden Soil (Temp. 37^0c and P^H 9.0)

Centrifugation and take supernatant

Sr.No.	Particulars	ml	ml
1	Supernatant	0.5	0.5
2	Distilled Water	7.0	7.0
3	Nesslers reagent	1.0	1.0
	Incubate at 37^0c for 10 min.		
4	Optical density	0.760	-

(Source :……..)

Table No. 4 : Farm Soil (Temp. 37^0c and P^H 7.0)

Centrifugation and take supernatant

Sr.No.	Particulars	ml	ml
1	Supernatant	0.5	0.5
2	Distilled	7.0	7.0

	Water		
3	Nesslers reagent	1.0	1.0
	Incubate at 37^0c for 10 min.		
4	Optical density	0.890	-

(Source :……..)

Table No.5 : Farm Soil (Temp. 37^0c and P^H 9.0)

Centrifugation and take supernatant

Sr.No.	Particulars	ml	ml
1	Supernatant	0.5	0.5
2	Distilled Water	7.0	7.0
3	Nesslers reagent	1.0	1.0
	Incubate at 37^0c for 10 min.		
4	Optical density	0.772	-

(Source :……..)

CALCULATIONS:

The activity of L-asparaginase units / mg calculate by

$$\text{L-asparaginase U/ml} = \frac{\text{Micromoles of ammonia released}}{\text{Incubation period} * \text{ml of cell suspension}} * 10$$

Farm Soil : (Temperature 37^0 C and P^H 7.0)

$$\text{Farm Soil} = \frac{0.75}{10 \times 10} \times 10$$

$$= \frac{0.75}{10 \times 10} \times 10$$

= 0.075 units / mg.

Table No. 6 : Results from the Calculations:

Sr. No.	Isolates	Conditions		Production U/mg.
		Temp.	PH	
01.	Farm Soil	37 ^{0}c	7.0	0.075
02.	Farm Soil	40 ^{0}c	9.0	0.032
03.	Garden Soil	37 ^{0}c	7.0	0.072

0 4 .	Ga rde n Soi l	40 ^{0}c	9 . 0	0.37

(Source: Primary Data)

DISCUSSION AND CONCLUSION :

L-asparaginase enzyme is present in many animal, tissue, bacteria, plants and in the serum of certain rodents. As far as microorganisms as a source of enzyme. Concern bacteria, fungi and actinomycetes are good source of L-asparaginase.

Researcher had collected two soil samples from Latur region and isolated microorganisms from it for production of L-asparaginase.

L-asparaginase is used as a chemothereaputic agent for the treatment of various types of diseases. It also plays an important in amino acid metabolism.

As per observation table it was concluded that L-asparaginase production from soil isolates for garden and farm soils maximum 0.075 at P^H 7.0 and incubation temperature 37^0c.

However it was concluded that the production of L-asparaginase from garden and farm isolates at P^H 9.0 and incubation temperature 40^0c. was produced at lower rate 0.032 and 0.037.

It was found that optimum P^H between 7.0 and 8.0 at 37^0c for enzyme production from garden and farm soil isolates.

Optimization of enzyme with reference to the different parameter like P^H, temperature and culture condition was found to be at P^H 7.0 at 37^0c from both soil isolates at compare to P^H 9.0 at 40^0c.

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