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Original Article

Small Scale Laboratory Culture Method Production and Characterization of L-Glutamic Acid by E.coli

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ABSTRACT

G*lutamic acid is one of the major amino acid produced by microbial fermentation provides 90% of world's total demand, and remaining 10% is met through chemical methods. It is the principal excitatory neurotransmitter in the brain and an important intermediate in metabolism. Present paper discuss the small scale laboratory culture method for production of glutamic acid. It was carried out by submerged fermentation technique where three different production media was prepared, sterilized, inoculated with the 24 hrs freshly prepared culture of E.coli. Production media was optimized with the selection of suitable carbon, nitrogen, minerals, vitamins, dyes which were required for the development of bacterial cells. Production medium was adjusted to pH7.0 with 1N HCl or 1N NaOH, kept in an orbital incubator shaker at 30°C at 120 rpm for 48 hr. End of the fermentation the cells and debris were removed by centrifugation at 10,000 rpm for 10 mts and supernatant solution was subjected to filtration. The filtrate was collected and dried to get the dry powder of glutamic acid. Progressive results and maximum yield of L- glutamic acid obtained with medium three and it was conformed with both chemical and analytical tests. L-glutamic acid produced by medium three showed positive results for ninhydrin and proved conformed result with xanthoproteic Test, UV spectroscopy and IR spectroscopy. This method can be applied in daily laboratory intended applications and these studies can be further extended for development of industrial techniques.*

Keywords: L-Glutamic Acid, E.coli, microbial fermentation, ninhydrin

1. INTRODUCTION:

Glutamic acid (symbol Glu or E) is an α-amino acid that is used by almost all living beings in the biosynthesis of proteins. It is non-essential in humans, meaning the body can synthesize it. It is also an excitatory neurotransmitter, in fact the most abundant one, in the vertebrate nervous system. It serves as the precursor for the synthesis of the inhibitory gamma-aminobutyric acid (GABA) in GABAergic neurons.

Glutamic acid was discovered by Ritthausen in 1866. In 1908 professor Ikeda found that the material enhancing the taste of sea weed (tangle) soup came from a sodium salt of glutamic acid (monosodium glutamate). Since then monosodium glutamate has been widely used in both the food industry and by the general public as a flavor-enhancing agent. The demand of glutamic acid has increased rapidly. Before 1956 glutamic acid was mainly obtained by the hydrolysis of plant proteins, e. g. gluten. In 1956 Japanese investigators reported the possibility of producing glutamic acid by microbiological methods, and began on an industrial scale the production of this amino acid. Because of their success, this method has been accepted by other nations, and also has stimulated investigators to find other micro-organisms and methods to produce glutamic acid more effectively. Though the recent interest in the manufacture of glutamic acid has shifted to chemical synthetic methods, the microbiological method of producing glutamic acid accounts for nearly all of the glutamic acid used today. Despite the widespread use of the microbiological method for the production of glutamic acid little has been published until recently on the conditions required for its production. In biotechnological processes, Corynebacterium species are used for economic production of glutamic acid by submerged fermentation. L-glutamic acid is produced per year using coryneform bacteria. A number of fermentation techniques have been used for the production of glutamic acid . Glucose is one of the major carbon sources for

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production of glutamic acid. Glutamic acid was produced with various kinds of raw materials using sub-merged fermentation of palm waste hydrolysate, cassava starch , sugar cane bagasse and date waste. In the present study L-glutamic acid is produced by E.coli using different fermentation media aiming maximum yield for industrial production of L-glutamic acid.

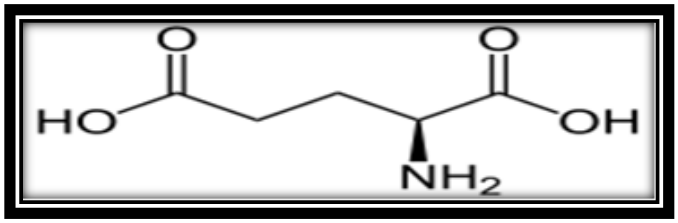

Fig 1: Structure of L-glutamic acid

2. MATERIALS AND METHODS

Media and Chemicals

All media components of high purity were obtained from Hi Media laboratories private limited, Mumbai, India. The remaining of all ingredients used was of analytical grade and the ingredients were purchased from SD fine chemicals limited, Mumbai, India. All media and chemicals were used without any pretreatment.

Equipment's

a. Orbital shaker incubator.
b. Rotator shaker
c. Centrifuge
d. Autoclave

Apparatus

a. Conical flask
b. Test tubes
c. Beaker
d. Bunsen burner

Method of Preparation

Procurement of Bacterial Culture and Preparation Of Inoculum:

Stock culture of E.coli was obtained from Department of Microbiology, St .Francis College, and Hyderabad. Inoculum was prepared by transferring cells from agar slant in to 250 ml flask containing 100 ml of the culture medium. Half of the culture was taken and inoculated in the production medium.

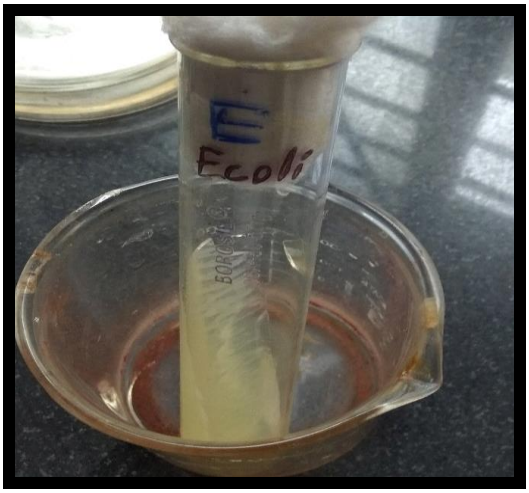

Fig 2: E- coli agar slant

Agar Slant and Culture Medium:

The constituents of the medium for preparing agar slant was kept at pH=7.0 and incubated at 30oC for at least three days 26. The slants were preserved at 4oC and sub cultured twice in a month.

Preparation of Fermentation Media 1:

Weighed chemicals dextrose of 5gms, magnesium chloride of 0.75 gms, urea of 0.5gms, potassium dihydrogen phosphate of 0.75gms, calcium chloride of 0.75gms, sodium carbonate of 0.25gms, valine 0.1mg. Were transferred into conical flask of 250 ml and contents were dissolved using distilled water , make up to 250 ml. The pH is maintained at 7. Now close flask with cotton and coil with aluminum coil, next autoclave the composition for 20 minutes at 1210 c and 15 lbs .

Table I: Composition of fermentation media formula 1

CHEMICALS	QUANTITY
Glucose	2gm
Potassium hydrogen phosphate	1gm
Magnesium sulphate	0.1gm
Ammonium sulphate	3gm
Calcium chloride	2gm
Sodium chloride	2gm
EDTA	0.2gm
Phenol red	Drop
Alanine	0.1mg
Vitamin B12	0.1mg

2. Preparation Fermentation Media 2:

Weighed chemicals lactose of 5gms, EDTA of 0.25 gms, potassium dihydrogen phosphate of 0.75gms, ammonium sulphate of 1gm, calcium chloride of 0.50gms, magnesium sulphate of 0.75gms, sodium chloride of 0.75gms, tyrosine

0.1mg. Were transferred into conical flask of 250 ml .and contents were dissolved using distilled water. And make up to 250 ml. The pH is maintained at 7. Now close flask with cotton and coil with aluminum coil .next autoclave the composition for 20 minutes at 120 c and 15 lbs.

Table 2: Composition of fermentation media formula 2.

CHEMICALS	QUANTITY
Dextrose	2gm
Potassium hydrogen phosphate	1gm
Magnesium chloride	0.1gm
Ammonium chloride	3gm
Meat extract	2gm
Calcium chloride	3gm
Sodium chloride	2gm
Urea	4gm
Methyl red	Drop
Vitamin b12	0.1mg

3. Preparation of Fermentation Media 3:

Weighed chemicals lactose of 5gms, calcium carbonate of 1gm, ammonium nitrate of 0.75gms, magnesium chloride of 0.25gms, sodium sulphate of 0.25gms, potassium dihydrogen phosphate 0.75gms, and alanine of 0.1mg. were transferred into conical flask of 250 ml. Contents were dissolved using distilled water. And make up to 250 ml. The pH is maintained at 7. Now close flask with cotton and coil with aluminum coil .next autoclave the composition for 20 minutes at 120 c and 15 lbs.

Table 3: Composition of fermentation media formula 3.

CHEMICALS	QUANTITY
Lactose	2gm
Potassium hydrogen phosphate	1gm
Magnesium chloride	1gm
Ammonium nitrate	3gm
Yeast extract	2gm
Calcium carbonate	3gm
Sodium sulphate	2gm
Methyl orange	Drop
Valine	0.1mg
Vitamin b12	0.1mg
Water	500ml

Fig 3: Production medium

Inoculation of Micro Organism into Media:

After sterilization the fermentation medium was inoculated with freshly prepared cultures of E.coli. The production medium was kept in an orbital incubator shaker at 30°C at 120 rpm for 48 hr.

Purification of the Compound:

After production for 48 hr the cells and debris were removed by centrifugation at 10,000 rpm for 10 mts and supernatant solution subjected to filtration. The filtrate was collected and dried to get the dry powder of glutamic acid.

Fig 4: Compound obtained in medium 3

3. RESULTS AND DISCUSSIONS

Yield Obtained By Fermentation Medium:

a. Yield obtained by Production medium: 1 is 500mg.
b. Yield obtained by Production medium: 2 is 900mg.
c. Yield obtained by Production medium: 3 is 2gm.

Report:

Highest yield was obtained in Production medium 3.

Identification and Characterization of Glutamic Acid

After centrifugation and filtration qualitative and quantitative analysis of glutamic acid is also an important point of consideration to obtain yield of the product. These are some methods used to determine glutamic acid.

1. Solubility Tests

Procedure:

a. Noted the solubility of amino acids in water and alcohol by placing a small amount in a test tube, adding a few mL of solvent and warming if necessary.
b. Determine the amino acid solution is acidic or basic by using a litmus paper while testing the solubility in water.
c. Repeat the solubility test using dilute HCl and dilute NaOH.

Report:

It showed a clear solubility in water, acid .It showed a cloudy solution in base or alkaline medium

Fig 5: HCl reaction

Fig 6: NaOH reaction

2. Ninhydrin Test

Procedure:

a. Prepared 1% of glutamic acid solution in distilled water
b. Added few drops of 2%ninhydrin solution to glutamic acid solution.
c. Boil the solution over a water bath for 2 min.

Report:

Development of blue colour or violet colour was obtained. Indicates positive test for amino acids.

Fig 7: Ninhydrin test

3. XANTHOPROTEIC TEST

Procedure:

a. Two ml of amino acid solution in a boiling test tube was added to equal volume of concentrated HNO3.
b. The solution was heated over a flame for 2 min and cooled thoroughly under the tap water.
c. Added 40% NaOH to make the solution strongly alkaline.
d. Solution was changed to brown colour indicates presence of glutamic acid.

Report:

Solution was turned to brown colour .Indicates positive test for amino acids.

Fig 8: Xanthoproteic test

Fig 9: Paper chromatography

4. PAPER CHROMATOGRAPHY

Materials Required:

a. 2% solution of individual amino acid
b. Solvent mixture of normal butanol acetic acid and water in 4:1:5 ratio
c. Ninhydrin solution

Apparatus:

a. Whatman's filter paper
b. Thread
c. Chromatography chamber
d. Capillary tube
e. Beaker
f. Reagent spray bottle

Procedure:

a. Taken whatmann's paper and marked a line at down of paper by using pencil. And placed the sample on line as a drop.
b. Now placed the paper in mobile phase 4:1:5 (butanol ,acetic acid , water)
c. Paper was removed after mobile phase was travelled 75% of paper and dried.
d. It was sprayed with ninhydrin solution
e. There was formation of light purple colour spots, which was measured by using retention factor.

Report:

There was formation of light purple colour spots, which was measured by using retention factor.

Rf= Distance travelled by solute/Distance travelled by solvent

= 1.23/1.25 = 0.98 cm

5. THIN LAYER CHROMATOGRAPHY:

Materials Required:

a. 2% solution of individual amino acid
b. Solvent mixture of normal butanol, acetic acid and water in 4:1:5 ratio
c. Ninhydrin solution

Apparatus:

a. TLC plate
b. Chromatography chamber
c. Capillary tube
d. Beaker
e. Reagent spray bottle

Procedure:

a. The stationary phase was applied onto the plate uniformly and then allowed to dry and stabilize.
b. With a pencil, a thin mark was made at the bottom of the plate to apply the sample spots.
c. Then, samples solutions were applied on the spots marked on the line in equal distances.
d. The mobile phase was poured into the TLC chamber to a leveled few centimeters above the chamber bottom. A moistened filter paper in the mobile phase was placed on the inner wall of the chamber to maintain equal humidity
e. Now, the plate prepared with sample spotting was placed in the TLC chamber so that the side of the plate with the sample line was facing the mobile phase. Then the chamber was closed with a lid.
f. The plate was then immersed, such that the sample spots were well above the level of mobile phase for development.
g. Allowed sufficient time for the development of spots.

Report:

There was formation of light yellow colour spots, which was measured by using retention factor.

Rf= Distance travelled by solute/Distance travelled by solvent

= 1.56/1.6= 0.97 cm

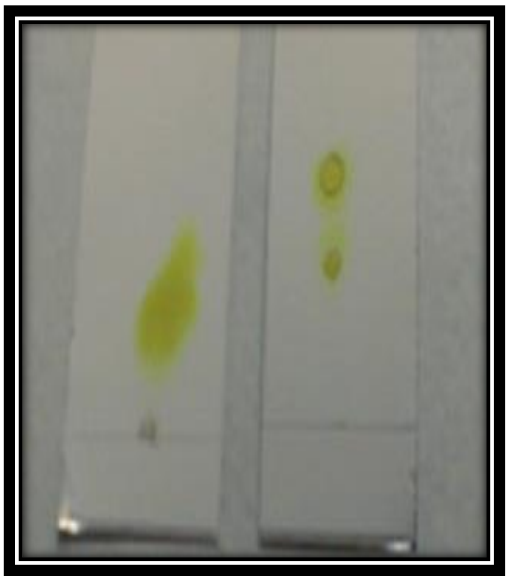

Fig 10: Spots obtained in Thin layer chromatography

UV SPECTROSCOPY

Procedure:

Performance of an Absorbance Spectrum

a. Filled the cuvette with the sample. To make sure the transfer is quantitative, rinse the cuvette twice with the sample and then fill it about ¾ full. Make sure the outside is clean of any fingerprints, etc.
b. Placed the cuvette in the spectrometer in the correct direction.
c. Covered the cuvette to prevent any ambient light.
d. A UV spectrum of glutamic acid was recorded by scanning between 200-400nm. From the spectrum glutamic acid showed maximum absorbance at 205nm which is similar to standard glutamic acid.

Report:

A UV spectrum of glutamic acid was recorded by scanning between 200-400nm. From the spectrum glutamic acid showed maximum absorbance at 205nm.

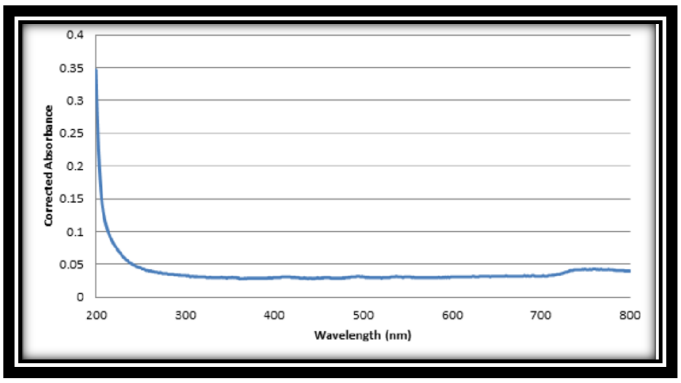

Fig 11: UV Spectra of Glutamic acid

IR SPECTROSCOPY

Procedure:

a. Turned on the IR spectrometer and allowed it to warm up.
b. Using a metal spatula, placed a small amount of sample under the probe.
c. Twist the probe until it locks into place.
d. Recorded the IR spectrum of the sample.
e. Recorded the IR spectrum of the standard glutamic acid
f. Repeated till obtain a good quality spectrum.
g. Recorded the absorption frequencies indicative of the functional groups present.
h. Cleaned the probe with acetone.
i. Turned off the spectrometer.

Report:

Analyze the obtained spectrum, shows the possible candidates for the sample, which is stated the probable identification of the sample.

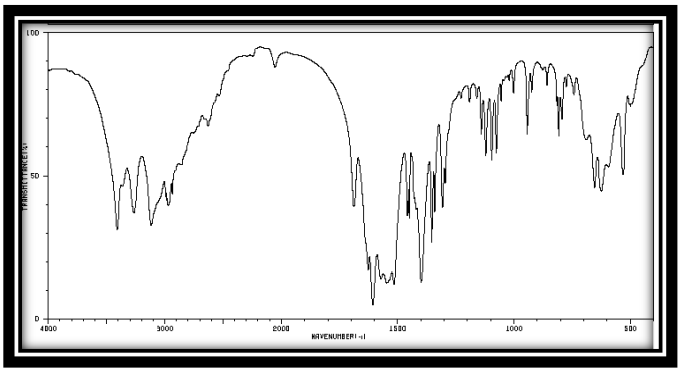

Fig 12: IR Spectra of Glutamic acid

4. CONCLUSION

a. L-glutamic acid production by means of fermentation methods, applying a variety of strains of micro organism obtained either by solid state fermentation and

submerged fermentation with different production mediums

b. Present work was attempted by production of glutamic acid by small scale laboratory culture method using microorganism E- COLI.
c. Progressive results and maximum yield of L- glutamic acid was obtained with medium three and it was conformed in both chemical and analytical tests.
d. L-glutamic acid produced by medium three showed positive results for Ninhydrin and given conformed result with Xanthoproteic Test, UV spectroscopy and IR spectroscopy.

A little work has been devoted to the fermentation process development and optimization still leaving large opportunities for further improvements in terms of improving productivity concentration and yield of glutamic acid.

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