



## Production and Crystallization of L-glutamic Acid

Dr. Dhurjeti Sarvamangala, Payala Vijayalakshmi\*

Gitam Institute of Technology, Gitam University, Rushikonda, Visakhapatnam-530 045, A.P, India.

Gitam Institute of Sciences, Gitam University, Rushikonda, Visakhapatnam -530 045, A.P, India.

\*Corresponding author's E-mail: [bavisettyvijayalakshmi2@gmail.com](mailto:bavisettyvijayalakshmi2@gmail.com)

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### ABSTRACT

The present invention contemplates a process of optimization of L-glutamic acid production by utilizing a novel substrate as carbon source i.e. starch tubers of *Cissus trifoliata* through submerged fermentation with bacterial and fungus cultures. Empirical optimization strategy was employed to evaluate the effect of various physico-chemical parameters on production. The highest glutamate production  $15.7 \text{ gl}^{-1}$  was recorded with *Corynebacterium glutamicum* DSM 20300<sup>T</sup> at pH 7.0, temperature  $30^\circ\text{C}$ , time 4d, urea  $2.0 \text{ gl}^{-1}$ , biotin  $2 \mu\text{gl}^{-1}$  and penicillin concentration  $5 \text{ Uml}^{-1}$ . Agitation at a speed of 120rpm was favourable for the production. Eventually the product produced was analyzed qualitatively by thin layer chromatography and quantitatively by standard ninhydrin method. The product purity was confirmed by Fourier transform infrared spectroscopy. Crystallization and testing of soil fungus for the glutamic acid production were the other key objectives of the present study.

**Keywords:** Glutamic acid, *Cissus trifoliata* tubers, submerged fermentation, optimization, crystallization.

### INTRODUCTION

Glutamic acid is an important flavoring agent. It has the largest commercial demand among the various amino acids. Besides that it occupies a pivotal position in a number of industrial processes such as food, pharmaceutical, medical, chemical industries etc. Glutamic acid reagents and its derivatives are used in protein synthesis techniques and with research in cell biology, biochemistry and biochemical processing methods. Therefore special effort has been devoted to the production of L-glutamic acid especially under the stand point of cheaper available raw materials to help go down the production costs.

The industrial production of aminoacids started with the availability of MSG in 1909. MSG was discovered in 1908 by Kikunae Ikeda and is responsible for one of the five basic tastes of the human sense of taste (umami). The production of L-glutamic acid through direct fermentation is 370,000 metric tons/annum<sup>2</sup>.

Literature survey by the authors of the current study has reported various methods of L-glutamic acid production<sup>10</sup>. The authors have also reported that fruits of *Mimusops elengi* L.<sup>11</sup> and *Muntingia calabura* L.<sup>12</sup> were the profitable substrates for the production of glutamic acid.

The present study is an attempt to unveil an inexpensive and novel fermentable substrate which has proven to yield appreciable amounts of glutamic acid. The plant *Cissus trifoliata* is a tuberous plant belongs to a family called vitaceae popularly known as possum – grape, sorrel vine or vine – sorrel which will crawl on the ground or just climbing with the use of tendrils. It is often called as Mandalamari by the village people.

### MATERIALS AND METHODS

#### Microorganisms

The bacterium *Corynebacterium glutamicum* DSM 20300<sup>T</sup> was obtained from the National chemical laboratory, Pune, India and *Arthrobacter globiformis* MTCC 4299 was procured from IMTECH, Chandigarh. The bacterial cultures were maintained on a basal nutrient agar slopes and stored in a refrigerator at  $4^\circ\text{C}$ . *Aspergillus niger* culture used in this study was isolated from soil sample through serial dilution technique and cultivated on potato dextrose agar at  $30^\circ\text{C}$  for 7d. The spore suspension ( $1 \times 10^7$ ) for inoculation was prepared by adding 10 ml of distilled water to two drops of 0.1% Tween 80 to the slant and carefully scraping the spores using an inoculating loop. Identification was done under microscope by using lactophenol cotton blue staining.

#### Substrate

In very large numbers, huge tubers of *Cissus trifoliata* were found and collected from the sea shore areas of Rushikonda beach, Visakhapatnam, India. The amount of starch present in the tubers was found to be 8.17 g per 100g of tuber sample as per the analysis report obtained from (VIMTA LABS PVT. LTD., Hyderabad, India). After starch acid hydrolysis<sup>6</sup> of tubers the amount of sugars released was estimated by phenol sulphuric acid method<sup>4</sup>. The Concentration obtained was  $21.30 \text{ gl}^{-1}$ .

#### Production medium and fermentation conditions

The study was performed at the laboratory scale in 250ml conical flasks containing 10ml of starch hydrolysate, 100ml of distilled water containing mineral salts. (urea:  $0.8 \text{ gl}^{-1}$ ,  $\text{K}_2\text{HPO}_4$ :  $0.08 \text{ gl}^{-1}$ ,  $\text{KH}_2\text{PO}_4$ :  $0.08 \text{ gl}^{-1}$ ,  $\text{MgSO}_4$ :  $0.04 \text{ gl}^{-1}$ ,  $\text{MnSO}_4$ :  $0.001 \text{ gl}^{-1}$ ,  $\text{Fe}_2(\text{SO}_4)_3$ :  $0.001 \text{ gl}^{-1}$ , Biotin:  $1 \mu\text{gl}^{-1}$ ) and 1% of inoculum or spore suspension was added to each



flask and after 24h of bacterial fermentation an aqueous solution of crystalline penicillin G was added at a concentration of  $1\text{Uml}^{-1}$ . However the addition of penicillin prior to 24h of bacterial fermentation would inhibit the production. The fermentation was carried out at varying physical conditions like temperature (25, 30, 35,  $40^{\circ}\text{C}$ )  $\text{p}^{\text{H}}$  (5.0, 6.0, 7.0, 8.0, 9.0), time (1 to 7d for bacterial fermentation and 1 to 11d for fungus fermentation) and chemical conditions like urea concentration (0.8, 1.2, 1.6, 2.0,  $2.4\text{gl}^{-1}$ ), biotin concentration (1, 2, 3, 4,  $5\text{ }\mu\text{gl}^{-1}$ ) and penicillin concentration (1, 5, 10, 15,  $20\text{Uml}^{-1}$ ).  $\text{P}^{\text{H}}$  adjustment was done by using 1N HCl or 1N NaOH.

### Crystallization

Two different methods were evaluated for the crystallization of glutamic acid from the culture filtrate. First, precipitation of glutamic acid at its isoelectric point ( $\text{pH}$  3.2) with 1N HCl, stored at  $20^{\circ}\text{C}$  for 48 h. By the Second method, 100ml of fraction filtrate was concentrated, hydrolysed with 20ml of 2N HCl and subjected to boiling for 20min. The hydrolysate was filtered and to the filtrate 20ml of 2% $\text{AgO}_2$  or  $\text{BaO}_2$  solution was added. Again after filtration supernatant was collected and subjected to boiling for  $60\text{-}70^{\circ}\text{C}$  for 15min. Upon rapid cooling at very low temperature for 1h crystal formation was takes place.

### Determination of glutamic acid

The fermented broth at the end of fermentation was subjected to centrifugation at  $10,000\times\text{g}$  for 10 min to remove microbial cells. Filtration can be done to remove mycelia mat. The collected supernatant was later subjected to thin layer chromatography (TLC) for qualitative identification of glutamic acid by using a solvent mixture containing 1-butanol, acetic acid, water in 80/20/20 (v/v) and adsorbent silica Gel G and running standard amino acids simultaneously on a chromatogram<sup>7</sup>. The concentration of glutamic acid was quantitatively estimated by standard ninhydrin method<sup>8</sup>. The product was finally confirmed by Fourier transform infrared spectroscopy.

## RESULTS AND DISCUSSION

The data represented in table 1 showed the production of L-glutamic acid at varying physico-chemical parameters. Varying all the parameters, standard conditions were established for optimum production by the three cultures. *C. glutamicum* DSM 20300<sup>T</sup> produce maximum concentration of L-glutamic acid  $15.7\text{mgml}^{-1}$  at  $\text{pH}$  7.0 ( $6.4\text{mgml}^{-1}$ ), temp  $30^{\circ}\text{C}$  ( $5.72\text{mgml}^{-1}$ ), time 4d ( $6.08\text{mgml}^{-1}$ ), urea  $2.0\text{gl}^{-1}$  ( $7.6\text{mgml}^{-1}$ ) biotin  $2.0\mu\text{gl}^{-1}$  ( $3.64\text{mgml}^{-1}$ ) and penicillin  $5\text{Uml}^{-1}$  ( $6.88\text{mgml}^{-1}$ ). *A. globiformis* MTCC 4299 produce highest amount of  $13.9\text{mgml}^{-1}$  at  $\text{pH}$  7.0 ( $7.04\text{mgml}^{-1}$ ) temp  $30^{\circ}\text{C}$  ( $5.76\text{mgml}^{-1}$ ) time 5d ( $7.08\text{mgml}^{-1}$ ), urea  $2.0\text{gl}^{-1}$  ( $7.8\text{mgml}^{-1}$ ) biotin  $4.0\mu\text{gl}^{-1}$  ( $4.68\text{mgml}^{-1}$ ) and penicillin  $10\text{Uml}^{-1}$  ( $6.16\text{mgml}^{-1}$ ). The soil isolate *A.niger* produce  $8.06\text{mgml}^{-1}$  of glutamic acid at  $\text{pH}$  5.0 ( $6.6\text{mgml}^{-1}$ ), temp  $28^{\circ}\text{C}$  ( $7.0\text{mgml}^{-1}$ ) time 9d ( $7.08\text{mgml}^{-1}$ )

urea  $2.0\text{gl}^{-1}$  ( $5.76\text{mgml}^{-1}$ ) & biotin  $1\mu\text{gl}^{-1}$  ( $2.12\text{mgml}^{-1}$ ). However there was no effect of penicillin on *A.niger*. Agitation at a speed of 120 rpm and 100rpm were favorable for bacterial and fungus fermentation even if tested at varied speeds. The production was also tested between the values of  $\text{pH}$  7-8, temp  $30^{\circ}\text{C}$  –  $35^{\circ}\text{C}$  for bacteria and  $\text{pH}$  5-6, temp  $25\text{-}30^{\circ}\text{C}$  for fungus fermentations but there was no significant increase in the production and the readings were declined.

**Table 1:** Effect of physico-chemical parameters on Glutamic acid production

Fermentation conditions	Conc. of L-glutamic acid (mg/ml)		
	<i>C.glutamicum</i>	<i>A.globiformis</i>	<i>A.niger</i>
<b>pH</b>			
5.0	4.88	2.16	6.60
6.0	5.52	3.84	4.16
7.0	6.40	7.04	2.04
8.0	5.88	2.96	0.72
9.0	5.52	1.60	-
<b>Temp (<math>^{\circ}\text{C}</math>)</b>			
25	0.84	1.88	4.36
30	5.72	5.76	6.20
35	4.16	2.12	3.92
40	0.96	1.16	-
<b>Time (d)</b>			
3	4.68	3.52	-
4	6.08	4.52	-
5	5.60	7.08	-
6	5.16	4.16	-
7	3.71	-	2.44
8	-	-	4.68
9	-	-	7.08
10	-	-	3.84
11	-	-	2.16
<b>Urea (g/l)</b>			
0.8	2.76	2.48	3.24
1.2	3.28	3.68	3.76
1.6	3.20	4.68	4.16
2.0	7.60	7.80	5.76
2.4	4.36	7.44	4.72
<b>Biotin (<math>\mu\text{g/l}</math>)</b>			
1	3.00	1.80	2.12
2	3.64	2.88	1.00
3	3.20	3.60	-
4	2.92	4.68	-
5	2.72	2.12	-
<b>Penicillin (U/ml)</b>			
1	5.08	3.00	-
5	6.88	5.12	-
10	6.08	6.16	-
15	5.64	3.44	-
20	5.12	4.60	-



The result of TLC was observed as a visible purple coloured spot having similar Rf value (0.34) similar to that of standard sample (0.35). The obtained clear crystals after cooling were then subjected to Fourier transform infrared spectroscopy (Dept. of organic chemistry, A.U., Visakhapatnam). The infrared spectra of the test sample indicated that the peak wave number values of all functional groups ( $3061.49\text{ cm}^{-1}$  for N-H,  $1613.75\text{ cm}^{-1}$  for C=O,  $2742.03\text{ cm}^{-1}$  for C-H) exhibiting a similar frequency to standard values of glutamic acid (Fig.1). The detailed I.R. spectrum serves as the finger print of the glutamic acid molecule.

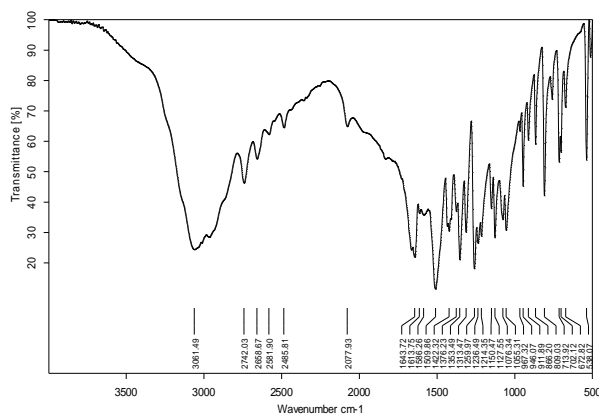


Figure 1: Infrared spectrum of glutamic acid

In recent years, there has been a rapid development of glutamic acid production by microbial fermentation. Large number of bacteria is now known to produce glutamic acid on commercial scale<sup>1</sup>. The previous studies had concluded that glutamic acid in substantial amounts have been produced by using fungal cultures like *Cephalosporium* species<sup>3</sup> and *Aspergillus* species<sup>9</sup>. Several carbohydrate materials were used as carbon sources like glucose, fructose, sucrose, maltose, molasses, starch hydrolysate, methanol etc. for the production of L-glutamic acid. In the past few years, an elaborative investigation has been done to establish the most pertinent and economically durable raw material for production. L-glutamic acid can be produced from cassava starch using *Brevibacterium divaricatum*<sup>5</sup>. Furthermore it was reported that it can also be produced from potato by *Brevibacterium linens*<sup>6</sup>. The present research was an attempt of using starch hydrolysate of *Cissus trifoliata* tubers as raw material and subjected to glutamic acid submerged fermentation using 'one-factor-at-a-time' method or empirical optimization strategy. This involves varying one factor while keeping the other factors unchanged under a specific set of conditions. The maximum yield obtained was  $15.7\text{ g l}^{-1}$  by *Corynebacterium glutamicum* DSM 20300<sup>T</sup>. The starch hydrolysate consists of  $21.3\text{ g l}^{-1}$  of sugars per 100g of substrate as per the analysis report obtained from spectrophotometric phenol sulphuric acid method read at 490nm. Nearly  $15.7\text{ g l}^{-1}$  of sugars out of  $21.3\text{ g l}^{-1}$  were converted to glutamic acid by *C.glutamicum*.

## CONCLUSION

The organic carbon source in any fermentation processes is the most expensive factor contributing to the cost of the process. In the current study, the carbon source is provided by the starchy raw material present in the *Cissus trifoliata* tubers an economical raw material produces high concentration of glutamic acid under optimum physico-chemical conditions. The information conveyed in this study will help in designing various schemes for scale up level production of L-glutamic acid which may prove to be an industrial asset.

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