

Production of L-Glutamic acid by *Corynebacterium glutamicum* DSM 20300^T and *Arthrobacter globiformis* MTCC 4299 using fruits of *Muntingia calabura* Linn.

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The present study was an illustrative investigation on L-Glutamic acid production by using two different bacterial strains like *Corynebacterium glutamicum* DSM 20300^T and *Arthrobacter globiformis* MTCC 4299 employing a cheaply available fruit substrate of *Muntingia calabura* L. Owing to the high sugar content of these fruits and quick establishment process of its plant an attempt was made to utilize the fruits for the production of glutamic acid, the precursor of mono sodium glutamate (MSG), employing submerged fermentation. In these experiments, optimization of fermentation parameters could be done using an empirical strategy method to determine key factor ranges for the production of glutamic acid. After optimization of physicochemical parameters, the highest production of glutamic acid was obtained 15.1 mg/ml with *Corynebacterium glutamicum* DSM 20300^T at p^H 7.0, temperature 30°C was maintained for 48h incubation time with urea, biotin and penicillin optimum concentration were being 2.0g/l, 1µg/l and 1U/ml respectively. On the other hand, the production of glutamic acid obtained with *Arthrobacter globiformis* MTCC 4299 was 10.7mg/ml at p^H 7.0, temperature 30°C time 48 h, urea 2.0g/l, biotin 4µg/l and penicillin concentration 15U/ml. Agitation at a speed of 120 rpm was favourable for the production. Eventually the product which was produced was analyzed by thin layer chromatography and subjected to crystallization at its isoelectric p^H 3.2. Finally the product presence was confirmed by Liquid chromatography-Mass spectroscopy.

Keywords: *Muntingia calabura* L., *Corynebacterium glutamicum*, *Arthrobacter globiformis*, Glutamic acid, Fermentation and Crystallization.

INTRODUCTION

Glutamic acid is of industrial importance because; it is widely used in food, pharmaceutical, medical,

Abbreviations

TLC: Thin layer chromatography

R_f: Retardation factor

Triple QQQ LC/MS: Triple Quadrupole Liquid chromatography/Mass spectroscopy.

M.calabura: *Muntingia calabura*

C.glutamicum: *Corynebacterium glutamicum*

A.globiformis: *Arthrobacter globiformis*

biochemical and analytical industries. Mono sodium glutamate (MSG), the sodium salt of glutamic acid is used commercially as a flavor enhancer, usually in combination with nucleotides inosinate to provide an expansion and extension of taste in processed foods. Besides the existence of efficient methods of industrial fermentation, many efforts are still on to improve the glutamic acid fermentation, particularly under the stand point of cheaper available substrates to help go down the production costs.

In the past few years, L-glutamic acid has been synthesized from a variety of raw materials through submerged fermentation. Literature survey revealed that, glutamic acid was produced from palm waste hydrolysate

supplemented with glucose using *Brevibacterium lactofermentum* ATCC 13869 Das et al. (1995). Several other agro waste materials were also used for the production of glutamic acid cassava starch Jyothi et al. (2005), sugar cane baggase (Amin and Al-Talhi, 2007) under submerged fermentation conditions. Later on, an investigation carried out by Mahmoud Tavakkoli et al. (2009) reported that a high amount of glutamic acid was excreted from date waste juice by *Corynebacterium glutamicum* CECT 690. Response surface methodology was used to optimize the submerged fermentation conditions. Glutamic acid can also be produced through solid state fermentation process (SSF). Sugar cane baggase enriched with 10% glucose liberated glutamic acid 80mg/g of dry sugar cane baggase (Nampoothiri and Pandey, 1996). However submerged fermentation was commonly used for the production of glutamic acid. The present method differed from all the other methods which had been reported so far, in that the fruit pulp of *Muntingia calabura* L. (*M. calabura* L.) was used as carbon source in order to get very higher yields of glutamic acid. The plant *M. calabura* L. (Elaeocarpaceae) usually known as Jamaica cherry or panama berry is a fruit species indigenous to Southern Mexico, Central America, Northern South America and the Greater Antilles, that has been distributed in south-east Asia and many other places as a road side adornment. In tropical lowlands it can sustain continuous growth. It tends to be an invasive plant since birds disperse the seeds and they germinate readily. The fruits have high nutrient value and rich in carbohydrate proportion (Janick and Paull, 2008). Tonnes of these fruits are being wasted or unutilized and containing a richest amount of sugars so conversion of these fruits to a useful product like glutamic acid is a novel thought.

One of the main intent of the current research was to optimize the basal nutrients and fermentation parameters like urea, biotin and penicillin concentrations, pH, temperature and time for getting high glutamic acid production using empirical optimization strategy or traditional 'one-factor-at-a-time' method. This involves varying one factor while keeping the other factors unchanged under a specific set of conditions.

Selection of suitable microorganisms is one of the most important stages of any fermentation process. In the present study, two different bacterial cultures like *Corynebacterium glutamicum* (*C. glutamicum*) and *Arthrobacter globiformis* (*A. globiformis*) have been chosen as they are high yielding strains of glutamic acid, biotin dependent and capable of growing in a natural media. Both are gram positive rods and produce white colour colonies on nutrient agar.

MATERIALS AND METHODS

Microorganisms

The bacterium *Corynebacterium glutamicum* DSM 20300^T was obtained from the National chemical laboratory, Pune, India and *Arthrobacter globiformis* MTCC 4299 was procured from IMTECH, Chandigarh and were used in this study. The bacterial cultures were maintained on a basal nutrient agar slopes and stored in a refrigerator at 4°C.

Medium used

The ripened fruits of *M. calabura* which contain rich quantity of sugars used to compensate the basic carbon requirements of the organisms. Urea was sterilized separately by filtration with Seitz filter and then added to the basal medium to supply the nitrogen needs of the culture. Other mineral salts K₂HPO₄, KH₂PO₄, MgSO₄, MnSO₄, Fe₂(SO₄)₃, biotin, penicillin were sterilized separately by autoclaving at 121°C for 15 minutes and added to the production medium.

Cultural conditions

10g of fruit pulp was dispensed separately in 250ml Erlenmeyer flasks containing 100ml of distilled water and mineral salts (urea: 0.8g/l, K₂HPO₄: 0.08g/l, KH₂PO₄: 0.08g/l, MgSO₄: 0.04g/l, Mnso₄: 0.01g/l, Fe₂(SO₄)₃: 0.001g/l, Biotin: 1µg/l) and 1% of inoculum was added to each flask and after 24h of fermentation an aqueous solution of crystalline penicillin G was added at a concentration of 1U/ml. However the addition of penicillin prior to 24h of fermentation would inhibit the production. The fermentation was carried out at varying physical conditions like temperature (20, 25, 30, 35, 40) pH (5.0, 6.0, 7.0, 8.0, 9.0), time (24, 48, 72, 96, 120h) and chemical conditions like urea concentration (0.8, 1.2, 1.6, 2.0, 2.4 g/l), biotin concentration (1, 2, 3, 4, 5 µg/l) and penicillin concentration (1, 5, 10, 15, 20 U/ml). P^H adjustment was done by using 1N HCl or 1N NaOH.

Analytical methods

Estimation of sugars in the fruit substrate

Preliminarily 100 mg of the fruit sample was weighed and subjected to acid hydrolysis with 5ml of 2.5 N HCl for 3h.

Later, the cooled hydrolysate was neutralized with Na_2CO_3 until the effervescence completely ceases and was subjected to centrifugation by adjusting the volume to 100ml. Subsequently the resultant supernatant was separated and subjected to various qualitative and quantitative tests for determination of sugars. Phenol sulphuric acid method Dubois et al. (1956) was used for the quantitative determination of sugars in the fruit sample.

Determination of glutamic acid

The fermented broth at the end of fermentation was subjected to centrifugation at $10,000\times g$ for 10 minutes to remove microbial cells. 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 (Sadasivam and Manickam, 2005). The concentration of glutamic acid was quantitatively estimated by standard ninhydrin method (Sasidhar Rao and Vijay Deshpande, 2005). The product was finally confirmed by Agilent 6410 Triple Quadrupole Liquid chromatography-mass spectroscopy (Agilent 6410 QQQ LC/MS) was used because it analyze the target compounds in complex matrices. In the mass spectra the desired compound produces a molecular ion peak located at m/z corresponding to its molecular mass. Agilent 6410 QQQ LC/MS utilize advanced nebulization technology and have three consecutive quadrupoles arranged in series to incoming ions.

The test sample for LC/MS was obtained by subjecting the fermented broth to crystallization at isoelectric pH 3.2 using 1N HCl and eventual drying in a vacuum desiccator yielded crystals of L-glutamic acid. The resultant granular crystals were visualized through microscopy.

RESULTS AND DISCUSSION

Glutamic acid has been the object of scientific study for over 150 years and has been sold as chemical product for the last eight decades. The highest produced amino acid approximately 9, 00,000 tonnes per year is glutamic acid which clearly demonstrates a proof of its increasing demand. Besides the existence of efficient methods of industrial fermentation, many efforts are still on to improve the glutamic acid fermentation, particularly under the stand point of cheaper available substrates to help go

down the production costs. In the past few years, an elaborative investigation has been done to establish the most pertinent and economically durable raw material for glutamic acid production.

A variety of cheapest saccharine materials like molasses, cane juice, beet juice, cassava roots, sago etc. are used for the production of glutamic acid Kono et al. (1965). Later on a research is carried out by Das et al. concludes that, palm waste hydrolysate is the best substrate which gives an elevated concentration of glutamic acid 88g/l using submerged fermentation. Despite, the existence of number of raw materials which fit into the above mentioned category, the present study introduces another novel substrate for glutamic acid fermentation- fruits of *M. calabura*. The plant *M. calabura* L. is an ever green tree found commonly on wastelands and its fruits become unutilized and left for natural degradation by soil microbial flora. These fruits were used as a substrates in the current research and subjected to glutamic acid submerged fermentation using OFAT method produces appreciable quantities of product i.e., 88.8% of glutamic acid higher than the yield acquired by the earlier studies on glutamic acid production from the substrates like sugarcane baggase (3.86%) by Jyothi et al., cassava starch (75.7%) by Amin et al., Mexican lime waste (13.7%) by Islas-Murguia et al. (1984), biotin rich beet molasses (55%) by (Haruo Momose and Takashi Takagi, 1978) , Palm waste hydrolysate (88g/l) by Das et al. The fruit sample consists of 17.0g of sugars per 100g of substrate as per the analysis report obtained from spectrophotometric phenol sulphuric acid method read at 490nm. Nearly 15.1g of sugars out of 17.0g were converted to glutamic acid. Qualitative sugar analysis reports of the fruit sample showed the presence of glucose, fructose and sucrose.

The entire experimental study was performed in Erlenmeyer flasks under aseptic conditions at varying physicochemical parameters using empirical strategy for the determination of the optimal cultural conditions to be maintained for the large-scale and real time fermentative production of glutamic acid. Glutamic acid production was analyzed qualitatively by TLC and quantitatively by ninhydrin method at various stages of fermentation by drawing minute samples intermittently during the process. The result of thin layer chromatography was observed as a visible purple coloured spot having similar R_f value (0.33) similar to that of authentic standard sample (0.35). The yield (%) was calculated as g of glutamic acid produced per 100g of sugar consumed in the fruit sample.

The data represented in various Figures 1^{a-f} showed the production of glutamic acid from the fruits of *M. calabura* L. by *C. glutamicum* and *A. globiformis* at varied fermentation conditions.

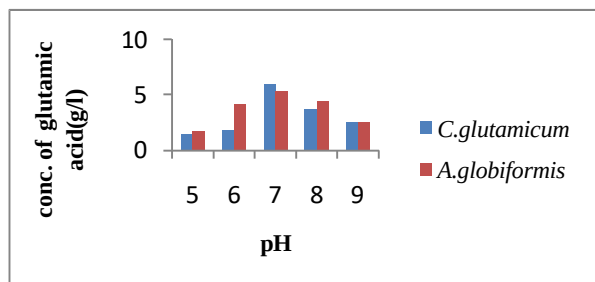


Figure1^a: Effect of p^H on glutamic acid production. Indicates that the optimum concentration of glutamic acid 5.88mg/ml for *C.glutamicum* and 5.28mg/ml for *A.globiformis* was recorded at p^H7.0

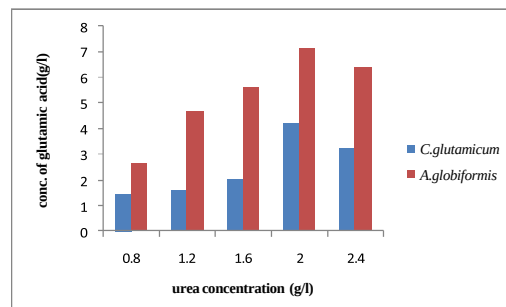


Figure1^d: Effect of urea concentration on glutamic acid production. Indicates that the concentration of urea 2% (W/V) leads to maximal concentration of glutamic acid 4.20mg/ml and 7.08mg/ml by *C.glutamicum* and *A.globiformis*.

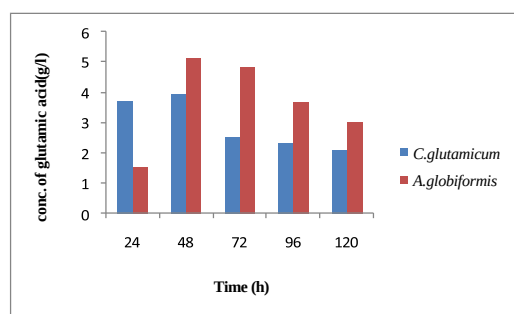


Figure1^b: Effect of time on glutamic acid production. Maximum production 3.92mg/ml and 5.12mg/ml was obtained with *C.glutamicum* and *A.globiformis* at 48h of fermentation with different time intervals.

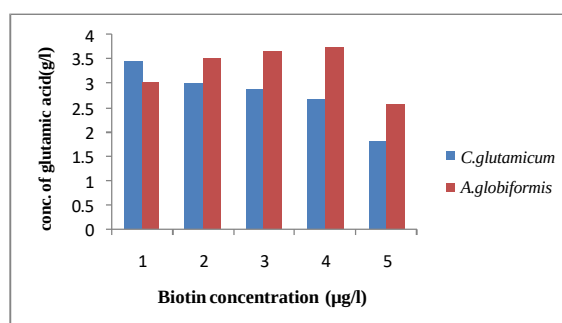


Figure1^e: Effect of biotin concentration on glutamic acid production. Shows the effect of biotin concentration on glutamic acid production 3.44mg/ml from *C.glutamicum* and 3.72mg/ml from *A.globiformis* with optimum biotin concentration 1 µg/l and 4µg/l.

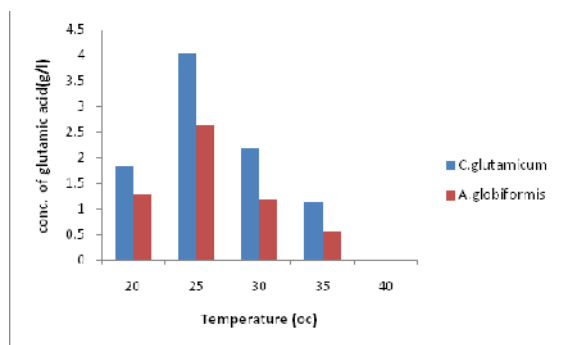


Figure1^c: Effect of temperature on glutamic acid production. Shows the effect of temperature on optimum production of glutamic acid 4.04mg/ml and 2.64mg/ml by *C.glutamicum* and *A.globiformis* at 30°C temperature.

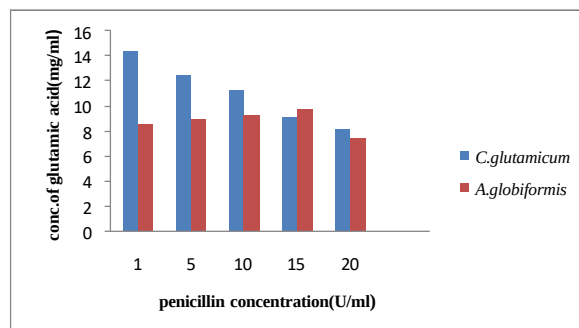


Figure1^f: Effect of penicillin concentration on glutamic acid production. Penicillin was one of the major critical factors on L-glutamic acid production. Maximum concentration of glutamic acid 14.3mg/ml by *C.glutamicum* and 9.8mg/ml by *A.globiformis* was achieved when the penicillin was added at a concentration of 1 U/ml and 15U/ml to the production medium

Effect of p^H

The influence of p^H was studied carefully on the yield of glutamic acid by subjecting the fermentation flasks to N varying p^H values ranging from 5.0-9.0 by the addition IN

IN HCl/1N NaOH. Consequently it was observed that p^H 7.0 favours the maximum yield of glutamic acid in both *C.glutamicum* (34.5%) and *A.globiformis* (31.05%). The production was also tested from p^H 7.1-7.9 but the readings were declined beyond p^H 7.0

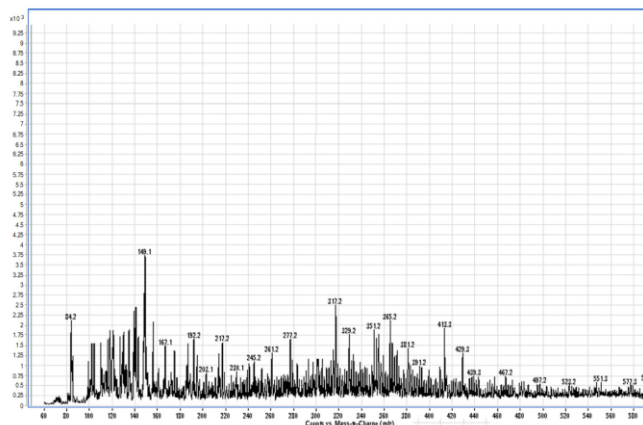


Figure 2: Mass spectrum of glutamic acid with *C. glutamicum* culture fermentation

Effect of time

The fermentation process was allowed to operate till 120h and the broth was periodically examined every 24h for maximum production. Fermentation efficiency of *C. glutamicum* (23.05%) and *A. globiformis* (30.1%) was observed to be high at 48h which gradually decreased upon further incubation.

Effect of temperature

Optimum temperature was a pre requisite for the opulent yield of glutamic acid. So, initially the production was tested under various incubation temperatures. In case of both the organisms, 30°C were found to be the ambient temperature yielding (23.7%) and (15.5%) for *C. glutamicum* and *A. globiformis* respectively.

Effect of urea

Urea was found to be the best and cheapest nitrogenous source for glutamic acid production. So, the study included determination of appropriate concentration of urea which gave highest output of glutamic acid production. A concentration of 2.0 g/l of urea was found to be suitable for the fermentation process yield (24.7%) with *C. glutamicum* and (41.6%) with *A. globiformis*.

Effect of Biotin

Surplus production of glutamic acid by microorganisms was found to be due to the permeability change induced by limiting the supply of biotin required by the bacterium. The concentration of biotin must be suboptimal for growth. Biotin in very low amount prevents the growth of microorganisms and high level inhibits L-glutamic acid

production by feedback control. Biotin concentration of 1µg/l and 4µg/l were optimum for the excretion of glutamic acid (20.2%) and (21.8%) by *C. glutamicum* and *A. globiformis*.

Effect of penicillin

Owing to its lytic activity on the cell wall there by releasing glutamic acid into medium, penicillin was found to be the most critical factor in the cellular excretion of glutamic acid. Penicillin was added at frequent intervals of 6h till 48h to fermentation flasks. But the Maximum yield of glutamic acid was obtained 84.1% with *C. glutamicum* and 57.6% with *A. globiformis* by the addition of penicillin to the production medium only after 24h of fermentation at a concentration of 1U/ml to *C. glutamicum* and 15U/ml to *A. globiformis*.

After standardizing all the physicochemical parameters at optimum conditions of production (pH7.0, time48h, temp.30°C, urea2.0g/l, biotin1.0µg/l and penicillin1.0U/ml by *C. glutamicum* and pH7.0, time48h, temp.30°C, urea2.0g/l, biotin4.0µg/l and penicillin15U/ml by *A. globiformis*) the fermentation was done again to achieve maximum production of glutamic acid. The maximum yield obtained was 15.1 mg/ml (88.8%) with *C. glutamicum* and 10.7mg/ml (62.9%) with *A. globiformis*. So the present results revealed that significantly high quantity of glutamic acid was obtained from *C. glutamicum* fermentation.

Afterward the finished broth was exploited for obtaining glutamic acid crystals by adjusting the pH to isoelectric point of glutamic acid 3.2. Agilent 6410 QQQ LC/MS is used for the determination of molecular mass of glutamic acid. In the resultant two different mass spectra the molecular ion peaks were located at 149.1(Figure.2) for *C. glutamicum* and 148.9 (Figure.3) for *A. globiformis* which confirmed the presence of glutamic acid. More peaks were visible larger than the molecular ion peak due to solvent used or impurity or an isotope of the molecular

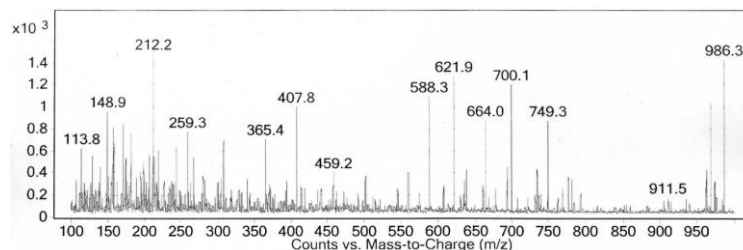


Figure 3: Mass spectrum of glutamic acid with *A.globiformis* culture fermentation

ion or may be due to the presence of more than one carbon atom in a compound. However peaks with mass less than the molecular ion were the result of fragmentation of the molecule. The mass spectroscopy of the samples was done at [Dept. of organic chemistry, Andhra University, Visakhapatnam].

CONCLUSION

The organic carbon source in any fermentation processes is the most expensive component contributing to the cost of the process. In the present study, the carbon source is provided by the sugars present in the fruits of *Muntingia calabura* Linn. 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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