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PRODUCTION OF L-GLUTAMIC ACID BY *ARTHROBACTER GLOBIFORMIS* MTCC 4299 USING FRUITS OF *MIMUSOPS ELENGI* LINN.

PayalaVijayalakshmi*, Dr. Dhurjeti Sarvamangala

*Gitam institute of technology, Gitam university, Rushikonda, Visakhapatnam-530045(A.P)

ABSTRACT: The aimed study is an illustrative investigation on L-glutamic acid production using inexpensive fermentable substrate-fruits of *Mimusops elengi* Linn. for the establishment of an economical fermentation process to meet the growing demands of L-glutamic acid in the global market at reasonable price. This profitable substrate produces opulent yield of L-glutamic acid under precise solid state fermentation conditions upon inoculation with *Arthrobacter globiformis* MTCC 4299. The production was tested at varying physico-chemical parameters like p^H, temperature, time, urea, biotin and penicillin concentration. The highest yield obtained was 87.5 g of L-glutamic acid per 100g of sugars consumed at p^H 5.0, temperature 30^0c upon 3d of incubation with urea, biotin and Penicillin optimum concentration as 2g/l, 3µg/l and IU/ml respectively. 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 and proton nuclear magnetic resonance spectroscopy. Finally the fermented broth was subjected to crystallization at the isoelectric point (p^H3.2) of L-glutamic acid using IN HCl.

Keywords: *Mimusops elengi* Linn.; *Arthrobacter globiformis* MTCC 4299; L -glutamic acid; Fermentation; Crystallization .

Abbreviations

FTIR : Fourier transform infrared spectroscopy

^{1}H NMR: Proton nuclear magnetic resonance spectroscopy

INTRODUCTION

In the past few years, L-glutamic acid has been produced by using variety of raw materials through submerged fermentation. L-glutamic acid can be produced from cassava starch using *Brevibacterium divaricatum* (Jyothi, et.al., 2005). An investigation was carried out on palm waste hydrolysate which produces best yield of glutamic acid by *Brevibacterium lactofermentum* ATCC 13869 (Das, et. al., 1995). Furthermore it was reported that, glutamic acid can be produced from sugarcane baggase using *Corynebacterium glutamicum* entrapped into carrageenan gel beads (Amin and Al-Talhi ,2007).A variety of cheapest saccharine materials like molasses, cane juice, beet juice, cassava roots, sago etc. have been used for the production of glutamic acid (Kono, et. al. ,1965).

The selection of a suitable substrate is a critical step in any fermentation process. Though much investigation has been made in this respect, but still much more inquisition is required. The present study is an attempt to unveil an economical and novel fermentable substrate which has proven to yield appreciable amounts of L-glutamic acid. The production satisfies the three key economic objectives in the fermentation stage-maximum yield, process productivity and substrate utilization.The fruits of *Mimusops elengi* Linn. produces higher concentration of L-glutamic acid. The plant is an ever green tree found commonly in South-east Asian countries as a roadside adornment. Due to its quick establishment process it grows well on waste lands.

Mimusops elengi Linn. belongs to sapotaceae family popularly known as bakula or Spanish cherry or bullet wood and it is well known for its ayurvedic medicine. The edible fruit is softly hairy becoming smooth, ovoid, bright red-orange when ripe. The fruit is a rich source of natural antioxidants and phenolic compounds (Chaiyan, et. al., 2009).The pulp of the fruit contains a large proportion of sugars and saponins (MisraandMitra,1967).
The organism used was *Arthrobacter globiformis* MTCC 4299 which produces optimum amount of L-glutamic acid under solid state fermentation conditions. Gram staining reveals that it is a short gram positive rod

MATERIALS AND METHODS

10 g of fully ripened fruit substrate which was collected from the local areas of Visakhapatnam, India was dispensed separately in 250ml Erlenmeyer flasks with 100ml of distilled water containing mineral salts 0.8 g/l urea, 0.08g/l K_2HPO_4, 0.08 g/l KH_2PO_4, 0.04 g/l $MgSO_4$, 0.001 g/l $MnSO_4$, 0.001 g/l $Fe_2(S0_4)_3$ and biotin 1µg/l to which 1% of *Arthrobacter globiformis* MTCC 4299(procured from IMTECH, Chandigarh, India) was added to each flask. Inoculum for fermentation was prepared by inoculating the culture from maintenance media into nutrient broth. The chemicals used were of analytical grade and sterilized separately. Urea and biotin sterilized through Seitz filters where as mineral salts by autoclaving at 121°c for 20 min. The fermentation was carried out at varying physical conditions like temperature (20°c,30°c,40°c,50°c,60°c), p^H (5.0,6.0,7.0,8.0,9.0), time (24h,48h,72h,96h, 120h) and chemical conditions viz urea concentration (0.8g/l, 1.2g/l, 1.6g/l, 2.0g/l, 2.4g/l) biotin concentration (1µg/l, 2µg/l, 3µg/l, 4µg/l, 5µg/l).p^H adjustment was done by using 1N HCL or 1N NaoH with p^Hmeter .However the fermentation was so effective under aerobic conditions in an orbital shaking incubator at 120rpm agitation. After optimizing the physico-chemical conditions penicillin was added at different concentrations of (1U/ml, 5U/ml, 10U/ml, 15U/ml and 20U/ml) to production media. The effect of Penicillin addition with time was also studied by periodical addition of penicillin with frequent time interval of 6h till 48 h.For the estimation of sugars in the fruit of *Mimusops elengi* Linn. initially 100mg of the fruit sample was taken and hydrolyzed with 5ml of 2.5 N HCl for 3h. The cooled hydrolysate was neutralized with sodium carbonate until the effervescence ceases. The neutralized sample was subjected to centrifugation by adjusting the volume to 100ml. The supernatant was separated and subjected to qualitative and quantitative test. For quantitative estimation of sugars Phenol sulphuric acid method was used (Dubois, et. al., 1956). After the completion of fermentation, the broth was heated and subjected to filtration and centrifugation at 10,000 rpm for 10 min to remove microbial cells. The resultant filtrate was subjected to thin layer chromatography for qualitative identification of L-glutamic acid by using a solvent mixture containing 1-butanol, acetic acid, water in 80/20/20 (v/v) and adsorbent silica Gel G (Sadasivam and Manickam, 2005). The produced glutamic acid was quantitatively estimated through standard ninhydrin method using ultraviolet-visible spectrophotometer (Sasidhar Rao and Vijay Deshpande, 2005) .The obtained supernatant was finally concentrated and 1N HCl was added to adjust the pH to 3.2 and then the medium was partially evaporated in a vacuum desiccator and allowed to stand still in a refrigerator until the glutamic acid crystals were precipitated there from. The obtained clear crystals were identified through binocular light microscope and subjected to Fourier transform infrared spectroscopy(FTIR) and Proton nuclear magnetic resonance spectroscopy(^{1}H NMR) for the of glutamic acid presence.

RESULTS

The data represented in various figures showed the production of glutamic acid from the fruits of *Mimusops elengi Linn*. by *Arthrobacter globiformis* 4299. The highest yield obtained was 87.5g/100g of sugars consumed from the fruit substrate upon addition of Penicillin at a concentration of 1U/ml to the production medium under optimized conditions. The yield (%) can be calculated as g of L-glutamic acid produced per 100g of sugar consumed in the fruit sample.

The preliminary product identification was done qualitatively by thin layer chromatography and quantitatively by spectrophotometric analysis. The result of the former test was observed as a visible purple coloured spot having similar R_f value 0.26 similar to that of authentic standard sample. The concentration of glutamic acid was estimated by standard ninhydrin method in the later test. The results were elucidated from (Fig.1[a-f])

Furthermore results of the FTIR spectrum showed in the (Fig.2) indicated that the peak wave number values of all functional groups (3431.18cm^{-1} for N-H ,1609.64cm^{-1} for C=O, 2925.89cm^{-1} for C-H) exhibiting a similar frequency to standard values of glutamic acid. The ^1H NMR (Fig.3) of glutamic acid showed four signals of varying size intensity. Although four signals obtained three corresponds to proton atoms of glutamic acid and largest signal 4.65 ppm is due to D_2O solvent in which glutamic acid crystals are dissolved.

DISCUSSION

The selection of raw material is very important in microbial fermentation and involves carbon sources that produce carbon skeleton for amino acids, and energy source for fermentable microorganisms. A detailed investigation is needed to establish the most suitable and economical raw material for L-glutamic acid production. Many different types of raw materials have been used for the purpose of L-glutamic acid production. Biotin rich beet molasses medium produce large amount of glutamic acid more than 55% by temperature sensitive *Brevibacterium lactofermentum* strain TS-88 (Haruo momose and Takashi Takagi ,1978).Later a high concentration of L- glutamic acid 88g/1 obtained from palm waste hydrolysate by *Brevibacterium lactofermentum* ATCC 13869 (Das, et. al.,1995). Dry sugar cane baggase yielded 80 mg glutamic acid/g when fermentation was carried out with *Brevibacterium* sp. (Nampoothiri and Pandey,1996) .Another study carried out on cassava starch hydrolysate which revealed that the strain *Brevibacterium* sp. DSM 20411 produced 25g/l of L-glutamic acid (Nampoothiri and Pandey,1999).

The present study is an approach in bringing into limelight an absolutely novel, cheaper and globally available substrate for glutamic acid production. The fruits of *Mimusops elengi* are rich in carbohydrates and serve as a salient substrate for glutamic acid production. The fruit sample consists of 16.9 g of sugars per 100g of substrate as estimated by phenol sulphuric acid method. The working principle of this method is that sugars undergo dehydration in the presence of sulphuric acid to furfural or hydroxyl methyl furfural that condense with phenol to form a yellowish orange coloured compound with an absorption maxima at 490nm. The various sugars present in the fruit sample were identified as fructose, glucose, lactose and sucrose by performing qualitative sugar analysis. *Mimusops elengi* L (sapotaceae) is an evergreen tree extending its distribution to many parts of the world, especially in the tropical forests of Asian and Australian continent. It is commonly grown as a shade tree in gardens and public places throughout the region. The entire production process was performed in Erlenmeyer flasks under sterile conditions and was tested at varying physico chemical conditions.

Effect of time: The L-glutamic acid concentration did not increase appreciably during the first 24h. The glutamic acid accumulation in the fermentation media slowly began later. Maximum yield of glutamic acid was recorded at 72h of incubation 40.4%. The L-glutamic acid yield then began to decrease and the lowest level reached was at 120h when only 13.9% was left in the fermentation media (Fig.1[a]). The optimum time for production was therefore found to be 3d.

Effect of temperature: An incubation temperature of 30^0c proved to be optimum for the growth of the bacteria and produce an yield of 13.49%. The medium with a temperature of 40^0c and above gave a decrease of L-glutamic acid production (Fig.1[b]).

Effect of p^H: The optimum p^H was found to be 5.0 for the initial stage of L-glutamic acid production by *Arthrobacter globiformis* from *Mimusops elengi* and the yield obtained was 28.4% (Fig.1[c]) Since urea was a main nitrogen source of the medium, the p^H rose initially and then dropped owing to the formation of acids from sugars. As the P^H dropped NaOH was added to keep the p^H from further decline.

Effect of urea: Urea was added to the fermentation media to compensate the nitrogen requirements of the bacteria. Results mentioned above suggested that the optimal amount of urea for L-glutamic acid accumulation was 2.0 g/l. This suggestion was supported by (Fig.1[d]) which indicates that the optimal yield of L-glutamic acid production 32.6% was found at 2.0 g/l.

Effect of biotin concentration: Excess production of L-glutamic acid by microorganism was found to be due mainly to the permeability change induced by limiting the supply of biotin required by the bacterium.(Fig.1[e]) indicates that the biotin at a concentration of 3µg/l produced 21.5% of glutamic acid.

The most important factor in the medium for the L-glutamic acid fermentation is biotin, which is an essential growth factor for the glutamic acid bacteria. The concentration of biotin must be sub-optimal for growth. Biotin in very low level prevents the growth of microorganism and high level prevents L-glutamic acid production by feedback control and by regulation of the cell – permeability barrier. When excess biotin is provided at the initiation by the fermentation, there is heavy cell growth, but lactic acid instead of L-glutamic acid becomes the principal product of the fermentation.

Effect of penicillin concentration: It was found that the maximum production was effected by maintaining optimum penicillin concentration 1U/ml owing to the lytic effect on cell wall of the bacteria which promotes the excretion of glutamic acid. Penicillin was added at frequent time interval of 6h till 48h to different flasks containing media. Maximum yield 87.5% was achieved when penicillin was added after 24h of fermentation at a concentration of 1U/ml. Penicillin was proved to be the major critical factor in glutamic acid production and this has been demonstrated by (Fig.1[f]).

After completion of fermentation, the broth was tested for the presence of L-glutamic acid by thin layer chromatography where the Rf values of the test sample approximately matched the standard run outs. The L-glutamic acid production was tested at varying physicochemical parameters. Each time the concentration of glutamic acid was estimated by ninhydrin method. Ninhydrin oxidizes the amino acid to aldehyde, releasing carbondioxide and ammonia. During the course of reaction, ninhydrin gets reduced to hydridantin. The hydridantin formed condenses with ninhydrin to yield a purple coloured complex. The crystals of glutamic acid were finally confirmed by FTIR and ^{1}H NMR. FTIR works on the principle that macromolecules possess a large number of atoms and as such may have numerous fundamental vibrations. The detailed I.R. spectrum serves as the finger print of the glutamic acid molecule. The infrared spectra of the test sample matched with the standards provided.NMR is used to identify nuclei based on the interaction of electromagnetic fields with a sample in a magnetic field. So FTIR identifies the functional groups and ^{1}H NMR identifies protonic groups of glutamic acid.

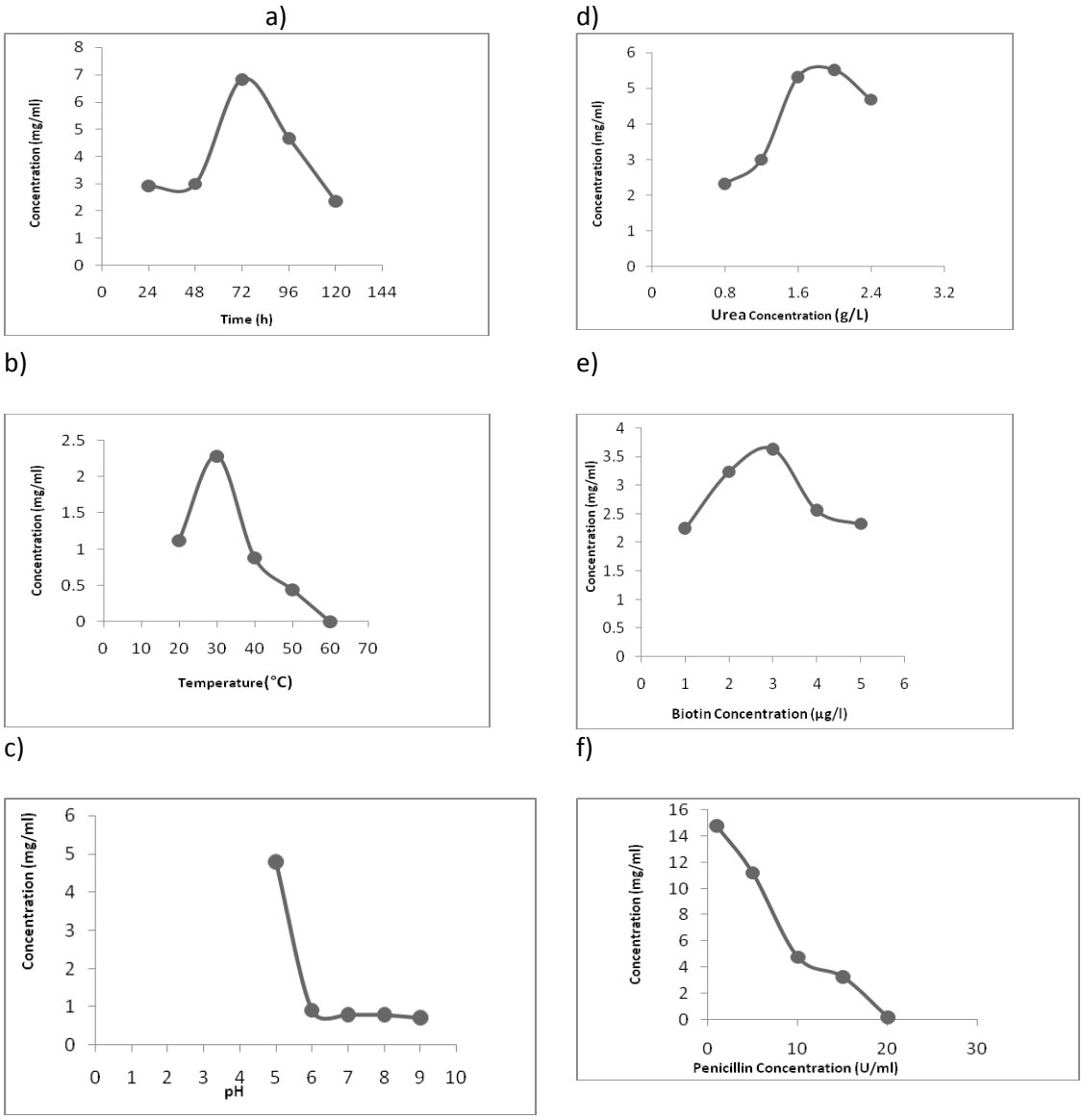

Fig.1[a] Shows the effect of time on glutamic acid production. Maximum L-glutamic acid concentration 6.84mg/ml was obtained at 72h of fermentation with different time intervals.
Fig.1[b] Shows the effect of temperature on optimum production of L-glutamic acid 2.28mg/ml at 30°c temperature.

Fig.1[c] Indicates that the optimum concentration of L-glutamic acid 4.8mg/ml was recorded at p^H-5.0 Fig.1[d] Indicates that a concentration of urea 2% (W/V) leads to maximal concentration of L-glutamic acid 5.52mg/ml.
Fig.1[e] Shows the effect of biotin concentration on glutamic acid production.3.64mg/ml of glutamic acid was produced with optimum biotin concentration 3µg/l. However the production of L-glutamic acid was decreased in proportion to increasing biotin concentration.

Fig.1[f] Shows the effect of Penicillin which was the major critical factor on L-glutamic acid production. Maximum concentration of glutamic acid 14.8mg/ml was achieved when the Penicillin was added at a concentration of 1 U/ml to the production medium.

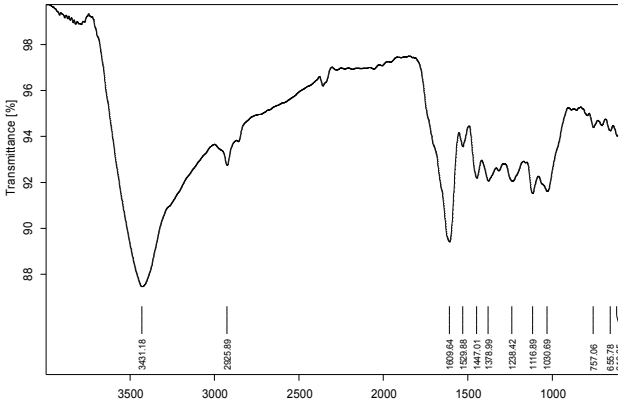

Fig. 2 Shows the infrared spectrum of glutamic acid.

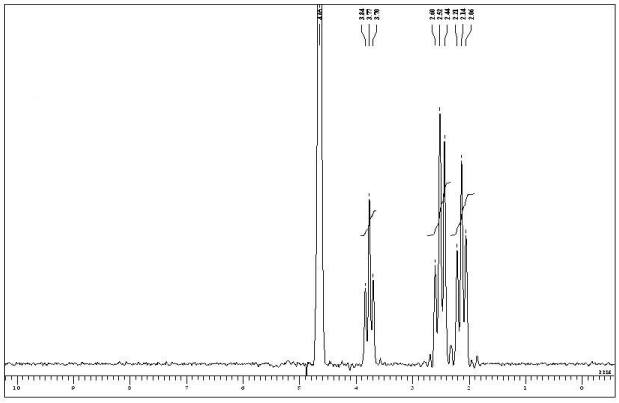

Fig. 3 Shows the ^{1}H NMR of glutamic acid.

The identification of L-glutamic acid was done by identifying the functional and proton groups noting the regions in which the peaks appear in the spectra. [Dept. of inorganic 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 *Mimusops elengi* L. 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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