

Biotin Auxotroph Mutants of Native *Corynebacterium glutamicum* for the Fermentative Production of L-Glutamic Acid

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The present paper deals with isolation of *C. glutamicum* strain and strain improvement of *C. glutamicum* by mutation studies for the production of L-Glutamic acid. Native isolate of *C. glutamicum* was manipulated to excrete L-glutamate. The modified protocol was used in this paper to trigger L-glutamate excretion. The important feature of this protocol is to achieve a biotin concentration that is low enough to activate glutamate excretion but high adequate to still allow sufficient growth of the cells. To achieve this appropriate biotin concentration, a pre cultivation step is required in order to deplete the cells of biotin. Later Physical Mutation was induced on Biotin depleted *C. glutamicum* strain.

Keywords: *Corynebacterium glutamicum*, L-Glutamic acid, Biotin, Fermentation.

Corynebacterium glutamicum was originally used as bacterium for the production of L-Glutamic acid¹. To achieve improved production of extracellular amino acid the changes in cellular metabolism are desired. L-Glutamic acid excretion by *C. glutamicum* is promoted by biotin limitation, treatment with penicillin, or by addition of fatty acid ester surfactants^{2,3,4}. Glutamate is often known as one of the primary products of nitrogen metabolism in the living cell and the glutamic dehydrogenase system correspond to an significant link between the metabolism of amino acids and carbohydrates. Glutamate is generally

transformed rapidly to various other amino acids and proteinaceous compound.

Glutamic acid was first produced in Japan by hydrolysis of wheat, gluten or Soya bean protein in 1908. The fermentation process was invented by Kyowa Hakko Kogyo in 1957, while Ajinomoto Company produced Glutamic Acid synthetically⁵. Commercial Glutamic Acid is made by fermenting molasses from sugar beet or sugar cane, a process similar to making wine, beer, sauce, and vinegar⁵. Higher levels of extracellular glutamate can induce seizures and excite toxic neuronal cell death. In glioma and other brain tumors, excessive glutamate free from tumor cells may be responsible for tumor associated necrosis and also possibly to capture caused due to peritumoral and tumoral brain tissue⁶.

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MATERIALS AND METHODS

Isolation of *Corynebacterium glutamicum*

Soil is a good source of microorganisms. Sewage contaminated soil samples were collected from local Anantapuramu sewage contaminated soil of Anantapuramu. The soil sample was serially diluted and plated on isolation medium and incubated for 48-72 hours at 30°C.

A modified Bouillon medium was used for isolation of *Corynebacterium glutamicum* which has the following composition⁸, (g/l) separately for each dilution. Peptone, 10.0, Meat extract, 5.0 Yeast extract, 2.0, Sodium chloride, 2.5 and Agar, 20.0. Ingredients were mixed in 1000ml distilled water and heated to dissolve completely. Cyclohexamide at a concentration of 50mg/l was added to restrict the growth of fungi⁹. The pH of the medium was adjusted to 7.0 by 0.1 N Sodium hydroxide, and sterilized by autoclaving for 15 minutes at 15 psi.

Pediococcus acidilactici NCIB-8018 (MTCC Customer Number 6650) which is a screening organism for glutamic acid producing bacteria was obtained from NCL, Pune. It was maintained on MRS Agar medium. *Corynebacterium glutamicum* ATCC 13032, a wild type culture was purchased from ATCC U.S.A. *Corynebacterium glutamicum* MH 20-22B microbial culture, a leucine auxotroph was procured from Professor Eggeling, Biotechnology Institute, Julich, Germany for academic purpose. *Corynebacterium glutamicum* ATCC 13032 and *Corynebacterium glutamicum* MH 20-22B, were maintained on nutrient agar medium.

Serial dilution

1 gm of sewage contaminated soil sample was dissolved in 10 ml sterile distilled water and dilutions were carried out. An aliquot of each dilution was transferred to a petriplate containing

growth media and spreaded. Triplicates were maintained for each dilution and incubated for 72 hrs at 30°C. Plates contained sufficient numbers (30-50) of discrete, well isolated colonies were selected as a source of culture to be evaluated for production of L-glutamic acid. The selected plates were termed as master plate.

Screening of *Corynebacterium glutamicum* strains

Medium of following composition was used for screening test⁹, Glucose 25.0g/l, Yeast extract 0.5g/l, Meat extract 0.5g/l, Ammonium sulphate 7.0g/l, Di-potassium hydrogen phosphate 7.0g/l, Potassium di-hydrogen phosphate 3.0g/l, Agar 20.0g/l and salts Magnesium sulphate. 7H₂O 0.52mg/l, Ferrous sulphate. 7H₂O 0.014mg/l, Manganese sulphate. 4 H₂O 0.005mg/l, Sodium chloride 0.010mg/l. 1.0 µg/l of Biotin used as a growth factor.

The screening method, which in essence represented a bioassay for glutamic acid, was carried out by replica plating bacterial isolates of various origins onto a series of plates containing screening medium. The replicated cultures were incubated for 72 hrs at 30°C. After growth, the cells on the test plates were inactivated by a strong dose of UV radiation; Detection of L-glutamic acid produced on screening medium by individual colonies was done by "Bio-autographic technique"⁹. The agar plates were overlaid with 10ml molten basal agar containing glutamate-auxotrophic bacterium, *Pediococcus acidilactici* NCIB-8018 (obtained from NCL, Pune). After incubation at 37°C, the test plates were scored for appearance of a halo growth of the assay organism around some of the UV-killed colonies. The glutamic acid-producers were then recovered from a non-UV-irradiated nutrient medium master plate. Pure cultures of positive colonies were developed by streak plate technique. These isolates were

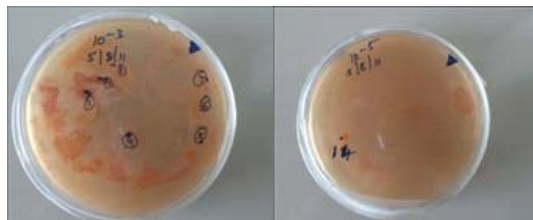


Fig. 1. Screening of glutamic acid producing microorganisms by Bioautographic technique



Fig. 2. *C. glutamicum* AB763933

subjected to preliminary morphological, cultural, and physiological and biochemical tests.

Production medium

Production media contain ingredients same as that of Screening medium without agar. 15 ml of production media was taken in 50 ml conical flasks and autoclaved at 15psi for 15 mins.

Cultivation of selected isolates

Each 15 ml production media containing flasks were inoculated with two loop fulls of selected isolates from slant culture tubes and incubated for 72 hrs at 30°C in orbital shaker at 200 rpm⁸.

Identification and characterization of selected isolates

Morphological characteristics

The selected isolates were subjected to Gram staining¹¹, spore staining and flagellation.

Cultural characteristics

Colonies obtained were allowed for culture tests such as growth, edge or surface structure, pigmentation.

Biochemical characteristics

The selected isolates were subjected to biochemical tests such as Catalase test¹¹, Carbohydrate fermentation test¹¹, Methyl red test and Voges-Proskauer test¹², Indole test, Hydrogen sulphide test¹², Phosphatase test, Urease test¹¹, Litmus milk test, Nitrate reduction test¹¹, Casein hydrolysis test¹², Starch hydrolysis test test¹¹, Gelatin liquefaction test.

Molecular Characterization of selected isolates

Isolation of DNA from suspected isolates

16s rDNA studies conducted to know the molecular characterization.

Biotin Auxotrophic mutants preparation

Biotin Auxotrophic mutants were prepared by following L Eggeling *et al*¹³. Preculture was done on LB agar plates and CGXII minimal medium and CGXII minus biotin medium are prepared.

Glutamic acid assay

The assay contains in a total volume of 1 ml: 850 μ l of buffer, pH 8.6 (75 mM Trisethanolamine, 10 mM KH₂PO₄, 0.5% Triton X-100), 10 μ l of 50 mM NAD⁺, 50 μ l of 1.5 mM iodinitrotetrazolium chloride, 10 μ l of diaphorase [350 U/ml], 10 to 100 μ l of sample (max. 25 μ M in assay), and 20 μ l of glutamate dehydrogenase (1,000 U/ml). The

reaction is acceptable to complete, it may take 20 min, and the absorbance is read at 492 nm against a blank without glutamate. The extinction coefficient for the formazan is 19.9 mM⁻¹ cm⁻¹. There are several commercial suppliers providing kits for quantitative L-glutamate determination.

Mutagenesis

In the present study fermentative production of glutamic acid was carried out by both wild type and mutants of *C. glutamicum*. Mutagenesis was carried out by physical and chemical mutagens. In the processes of Physical mutagenesis actively growing culture of *C. glutamicum* was inoculated on five culture plates by spread plate technique and the plates were exposed to Ultra Violet light (irradiated) with a dose of 300 erg/mm² for 30 sec, 60 sec, 90 sec, 120 sec and 150 sec respectively and incubated. The survivors of the mutagenesis were again subjected to a second dose of mutagenesis. Finally the survivors of second dose were used in this study.

Qualitative and quantitative estimation of glutamic acid

The fermentation process was monitored for 6 days during which samples were taken every 24 hrs. Instantly after sampling, pH of samples was measured and the centrifuged at 8000xg in order to separate the cells; supernatant was used for estimation.

Fermentation Media

The basal fermentation medium for glutamic acid production contained 36 g glucose, 1 g KH₂PO₄, 1 g MgSO₄ · 7H₂O, 2 ppm each of Fe²⁺ (as FeSO₄ · 7H₂O) and Mn²⁺ (as MnSO₄ · 4H₂O), 100 I g thiamine · HCl, 0.24 g soybean hydrolysate (as total nitrogen), 4 ml of 0.1 % cresol red solution and appropriate amounts of biotin and urea per liter of deionized water. pH was adjusted to 7.0 by KOH before autoclaving.

Fermentation Studies of *Corynebacterium glutamicum*

Two loops of *Corynebacterium glutamicum* cells were grown on nutrient agar strain maintenance plate for 24 hours at 30 °C were inoculated into 20 ml of Rich medium (Seed broth) in a 250 ml conical flask and cultivated for 24 hours in orbital shaker at 30 °C with 120 rpm. The Seed broth was transferred to 180 ml of Inoculum medium in an Erlenmeyer flask and was kept in orbital shaker at 30 °C with 120 rpm for 40 hours. This Inoculum

was used to inoculate the Fermentation medium in the Bioreactor and batch fermentation was carried out for 72 hours.

Qualitative analysis of L-glutamic acid

Thin layer chromatography was used for qualitative detection of glutamic acid. TLC plates of 0.2 mm thickness were prepared using adsorbent material silica gel. The fermented broth culture was centrifuged and supernatant was collected and the supernatant which served as source of glutamic acid. Supernatant spotted on TLC plates along with glutamic acid standard and developed in a solvent system containing a mixture of n-butanol, acetic acid and water (4:1:1:v/v) for about 6 hrs. After development the chromatogram was sprayed with 0.15% ninhydrin ethanol solution and was kept in oven at 110°C for 3 min⁸ spots of all amino acids were visualized. R_f value of the samples were compared with the R_f value of glutamic acid standard. The ninhydrin colour reaction¹⁴ was used for quantitative determination of L-glutamic acid where the absorbance was measured at 570 nm. 1ml of freshly prepared ninhydrin reagent was added to the equal volumes of supernatant and heated in water bath for 15 min. and cooled under running tap water. The absorbance was measured against blank without supernatant. The amount of glutamic acid present in the sample was estimated from the standard curve for L- glutamic acid.

RESULTS AND DISCUSSION

Isolation and identification *Corynebacterium glutamicum*

Corynebacterium glutamicum strains were isolated from sewage contaminated soils of Anantapuramu. The modified Bouillon medium was used for isolation⁸. Cyclohexamide 50mg/L was added in the medium⁹ to restrict the growth of fungi. Total 1500 bacterial isolates from sewage contaminated soils were isolated and tested for the L-glutamic acid production. The bacterial isolates producing L-glutamic acid were screened with the media containing carbohydrates, nitrogen source and supplemented with inorganic salts.

Lederberg and Lederberg 1952 reported Replica plating technique was used to transfer the organisms from isolation medium to screening medium and Bioautographic technique using

dehydrated L-glutamic acid assay medium and *Pediococcus acidilactici* NCIB -8018 as test organism. Following incubation at 37°C for 24hrs the test organism grows in the immediate area surrounding any given colony that produced L-glutamic acid. Eighty one (81) isolates out of fifteen hundred (1500) were found to produce L-glutamic acid. Eighty one isolates (81) showing positive results were subjected to purification in to the same medium to elevate their L-glutamic acid producing capability and to identify *Corynebacterium glutamicum*.

In preliminary morphological screening tests of isolates, Out of Eighty one⁸¹ isolates only fourteen¹⁴ isolates were observed to possess characteristic properties of Coryneform bacteria. These 14 suspected isolates of Coryneform bacteria were subjected to Morphological, cultural, bio-chemical, and physiological characteristics were recorded respectively. Out of 14 only 6 isolates were tentatively identified by taxonomic comparison of *Corynebacterium glutamicum*. These includes isolate no SKUDBT-6, SKUDBT-202, SKUDBT-490, SKUDBT-681, SKUDBT-878, SKUDBT-1226.

The plates were layered with basal agar medium for glutamic acid bio-assay containing the assay organism *Pediococcus acidilactici* NCIB-8018 dispersed in the medium. Halo of growth developed around the colony producing glutamic acid during incubation at 37°C. Colonies surrounded by halos on test plates were picked as glutamic acid producers.

Molecular characterization of isolates

The Basic Protocol, using phenol extraction and ethanol precipitation, is appropriate for the purification of DNA from small volumes of suspected bacterial samples. Equal volumes of isopropanol and DNA solution are used in precipitation. Note that the isopropanol volume is half that of the given volume of ethanol in precipitations. This allows precipitation from a large starting volume in a single micro centrifuge tube. Isopropanol is less volatile than ethanol and takes longer to remove by evaporation. Some salts are less soluble in isopropanol (compared to ethanol) and will be precipitated along with nucleic acids. Extra washings may be necessary to eliminate these contaminating salts.

16s rRNA gene was amplified from

purified genomic DNA with 16sF(5'AGAGT TTAGCCTG GCTCAG3') and 16sR (5'GGTTACCT TGTTACGACTT-3') primers. The amplified gene was sequenced by using Big Dye chemistry software. A260/280 ratio of the above DNA was recorded and the purity of the DNA was good.

Based on 16s rDNA partial sequence analysis with ChromasPro, Sequencing Analysis software the six strains showing similar characteristics of *C. glutamicum* and were the new variants of *C. glutamicum* and showing 92% similarity with *C. glutamicum* K051 strain (ATCC sub-strain K051). The 16s rDNA partial sequence was submitted to NCBI and got the Accession Number AB763933. 16s rDNA partial sequencing analysis of the remaining eight strains clears that they are new variants of *Bacillus flexus*, *Bacillus thuringiensis*, *Bacillus stratosphericus*, the sequences were submitted to NCBI with Accession Numbers AB686280, AB686281, AB701758.

CONCLUSION

The native isolate with accession AB763933 was converted into biotin auxotroph mutant. The native biotin auxotrophic mutant exposed to U.V mutation. Resultant U.V mutant was studied for its fermentation efficiency by using standard glutamic acid production media¹³. The strain yields 15.5 g/L, which is very slight increase of production with *C. glutamicum* improved strains. Further intensive molecular based strain improvement studies may be necessary to improve the L-Glutamic acid yield by using suitable fermentation medium and easy downstream process.

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