

Production of L-Arginine from arginine analogue resistant mutants of *Corynebacterium glutamicum*

Anjaneyulu Musini¹, Lakshmi Narasu Mangamoori², L. Said^{3*}

¹Centre for Biotechnology, Institute of Science & Technology, Jawaharlal Nehru Technological University, Hyderabad, INDIA

²Centre for Biotechnology, Institute of Science & Technology, Jawaharlal Nehru Technological University, Hyderabad, INDIA

³Centre for Biotechnology, Institute of Science & Technology, Jawaharlal Nehru Technological University, Hyderabad, INDIA

*Corresponding Author: lavudisaida@jntuh.ac.in

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Abstract- L-arginine is an essential amino acid, widely used in pharmaceutical, feed and food industry. It is the precursor molecule in citrulline and nitric oxide synthesis, which in turn plays significant role in a wide variety of patho - physiological functions in the cardiovascular, central and peripheral nervous systems. In the present study, mutagenesis experiments were carried out for isolation of analogue resistant mutants against *Corynebacterium glutamicum*. Initially, MIC (Minimum inhibitory concentration) was identified for arginine analogues by tube dilution method. MIC values found to be Arginine hydroxamate – 680 µg/ml. Further, mutagenesis of *C. glutamicum* was carried out with NTG (300µg/ml) and amino acid analogue resistant mutants were isolated in minimal media. Fermentation was carried out by resistant colonies and estimated the Arginine levels. Production level was found to be 5.2 mg /ml where as in control cells it was 1.4 mg /ml. This study highlighted the *C. glutamicum* mutant of L-arginine amino acid production.

Key words: L-Arginine, Mutagenesis, Minimum inhibitory concentration and *Corynebacterium glutamicum*

I. INTRODUCTION

The production of L-arginine is generally carried out by chemical synthesis or extraction of protein hydrolysates from L-ornithine produced by the process of fermentation. So far, a highly desirable method, the direct fermentation of L- arginine, is not available.

There are eight steps in the arginine biosynthesis pathway from glutamate, the first precursor of cycle. This pathway is regulated by the levels of arginine present in a wide variety of bacteria [1, 2, 3, 4, 5]. Arginine-requiring mutants produce large amounts of citrulline or ornithine or when arginine is limiting [6, 7] although their wild-type strains donot accumulate arginine due to these feedback controls by arginine. Arginine produced by mutants with a defective regulatory mechanism and that regulatory mutants could be found among the group of analogue- resistant mutants.

L-arginine biosynthesis was regulated by two regulatory patterns in different microorganisms [8]. *Corynebacterium glutamicum*, a glutamic acid producing bacteria, has advantage as an L-arginine producer because N-acetylglutamokinase, enzyme in the biosynthesis is subjected to feedback inhibition by L-arginine and also it carries

strong flux towards L-glutamate formation. Importantly, for the high yield of L-arginine, the repression of regulator gene (encoded by argR) in the operon coding L-arginine biosynthesis (arg) is considered as one parameter as well the feedback inhibition of N-acetylglutamate kinase as second parameter [9,10].

In the present study, glutamic acid producing bacteria, *C. glutamicum* was examined after treating with mutagen in the presence of amino acid analogue on minimal media. The amino acid production was found to be more in resistant mutant compared to wild strain.

II. MATERIAL AND METHODS

2.1 Bacteria culture:

Corynebacterium glutamicum strain was procured form MTCC, IMTEC, Chandigarh India. The culture was maintained on Mueller hinton agar slant at 4°C, revived in Mueller hinton broth and cultured at 37°C before their use.

2.2 Mutagenesis:

A loop full of the active culture was inoculated into the nutrient broth and incubated. 50 µl of the above grown

culture was added to each of the six sterile boiling tubes that contain 5 ml nutrient broth and incubated for 8 – 10 hours (until the O.D reaches 0.8) in a shaking incubator at 37°C and 200 rpm. Centrifuge the samples at 8000 rpm for 15 minutes. The cell pellet was dissolved in 5 ml citrate buffer (pH –5.5). Concentration of NTG used was 200-600 µg/ml. Incubate the tubes at 37°C in an incubator for 45 min. Collect the cell pellet and wash twice by using minimal A buffer (K₂HPO₄, KH₂PO₄, (NH₄)₂PO₄ and Sodium Citrate) solution. Finally the cell pellet was dissolved in 2 ml minimal buffer solution. 0.1 ml of each microbial suspension was serially diluted up to 10⁻⁴ dilutions. 0.1 ml of the above microbial suspension from each of the 10⁻⁴ dilution tube was spread plated onto the respective minimal media plate. The plates were incubated at 37 ° C for 24 hours. Numbers of colonies on each plate were counted and compared with that of the control plate. The concentration at which the number of colonies would be reduced to 50%, when compared to number of colonies on the control plate will be considered as optimized concentration. [11]

2.3 Fermentation:

Seed media: Glucose – 2%, Peptone – 1%, Yeast Extract – 1%, NaCl – 0.5%, initial p^H -6.52, Adjusted P^H – 7.2 (Before sterilization). 2% glucose was added to seed media, separately after sterilization. A loop full of cultures inoculated in to seed media. Incubation kept at 30°C, 200 rpm for overnight.

Production media: (NH₄)₂SO₄ -3%, KH₂PO₄ -0.05%, K₂HPO₄ – 0.05%, MgSO₄ – 0.025%, CaCO₃ -2%, Initial pH- 6.84, Adjusted P^H – 7.2 (Before sterilization). Glucose was sterilized separately, after cooling the media 10% glucose was added to production media. 4% inoculums of culture inoculated to production media. Incubation kept at 30°C, 200 rpm [12].

2.4 Estimation of arginine by Sakaguchi test:

Inoculated the isolated resistant colony into seed media and then into production media. Estimation of L-Arginine through Sakaguchi test was done for every 24 hours [13]. Arginine standard solution was prepared by dissolving 0.1gm arginine in 10 ml of sterile water. Different concentration range 4 - 36 µg was used for construction of standard graph. Double distilled water was added to make up the final volume to 4ml to which 1ml of KOH to each tube was added and mix gently. 2ml of 0.1% naphthalene was added and then 1ml of 40% urea was added and mixed gently. Tubes were kept at 4⁰ C for 10 min. 1ml Potassium hypobromite was added and gently mixed. O.D. values were taken by UV Spectrophotometer at 540nm. Standard Graph was constructed and concentration of Arginine in culture was checked at different time points.

III. RESULTS AND DISCUSSION

3.1 Mutagenesis:

Amino acid analogue arginine hydroxamate inhibited the growth by different bacteria and also reversed in the presence of arginine amino acid [14]. In order to isolate arginine analogue resistant mutant initially, the minimum inhibitory concentration of arginine hydroxamate was determined by tube dilution method. MIC of the arginine hydroxamate was found to be 683 µg/ml against *C. glutamicum* and same concentration was used for isolation of amino acid analogue resistant mutant. Strain improvements play an essential role in the commercial development of microbial fermentation [15]. In the recent years, new methods such as rationale screening and recombinant DNA technology have begun to make a significant contribution to this activity [16]. However, mutagenesis and random screening is still cost effective process and reliable short-term strain development is frequently the method of choice.

Chemical mutagenesis by methyl nitro nitrosoguanidine (MNG or NTG) is a successful method to the strain development for high yield production [17]. In the present study, NTG was used for mutagenesis experiment. The methyl nitro nitrosoguanidine (MNNG or MNG) mutagen concentration was optimized towards *Corynebacterium glutamicum*. Culture was treated with different MNG concentrations and calculated the survival rate of the organism. Graph of mutagenesis concentration verses survival rate of bacteria shown in Fig 1. 50% growth was inhibited at 300µg/ml MNG concentration, similar concentration used for the mutagenesis experiment for isolation of resistant colonies. The concentration at which the number of colonies would be reduced to 50%, when compared to number of colonies on the control plate will be considered as optimized concentration.

The concentration at which the number of colonies would be reduced to 50%, when compared to number of colonies on the control plate will be considered as optimized concentration. After treating with optimized concentration of MNG, culture was grown on minimal media containing arginine hydroxamate (893 µg/ml) and after incubation of few days three resistant mutant colonies were obtained shown on Figure 2.

After obtaining resistant mutants, colonies were grown on same analogue concentration in the minimal media for stabilization of mutants.

3.2 Fermentation and estimation of arginine

Fermentation was carried out at shake flask level by mutant colonies and wild strain. Culture was incubated over night in to the seed media and next transfers to the production media.

Seed media and production media composition given in materials and method section. Every 24 h interval culture sample was collected and arginine amino acid was estimated. Sakaguchi spectrophotometer method was used for analysis. Under alkaline condition α -naphthol react with guanidine group of arginine amino acid, which upon treatment with hypo bromate, produces characteristic red color. Arginine amino acid production from different resistant mutants and control wild strain was shown in figure 3.

After mutagenesis three colonies Cgarghx1, Cgarghx5 and Cgarghx10 were obtained and these three colonies are purified and checked for arginine production. Among all purified colonies Cgarghx 1 was produced more amino acid production (5.2mg/ml) at 72 h compared to the other mutant and in the wild strain only 1.4mg/ml arginine amino acid production. In the resistant mutant strain amino acid production was more due to regulatory abnormalities of the arginine biosynthetic enzymes [12].

IV. CONCLUSION

Literature has shown not many reports on L-Arginine amino acid production from *Corynebacterium glutamicum* till date. In the present study, amino acid analogue resistant mutant for production of L-arginine was isolated by classical mutagenesis. Amino acid production was observed to be 5.2 mg/ml at shake flask level fermentation. However, the future strategy would include the optimization of media components for higher yield.

V. ACKNOWLEDGEMENT

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VI. CONFLICT OF INTEREST

Author has no conflict of interest.

VII. AUTHORS' CONTRIBUTIONS

MLN has given the concept and designed the experiments, LS has monitored the experimental work, AM has carried out the experiments.

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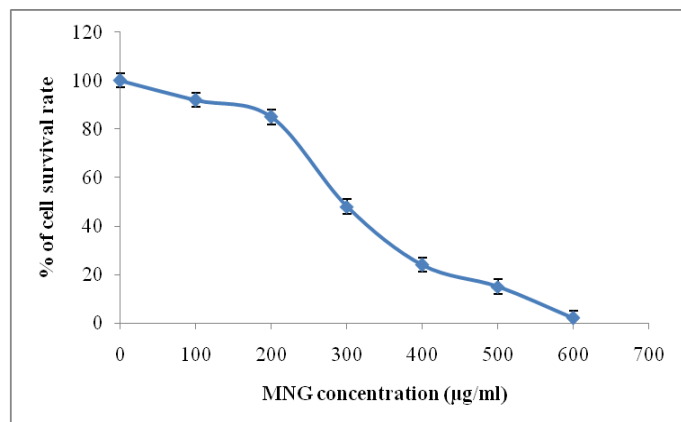


Figure 1: Survival rate of *C. glutamicum* with MNG at different concentrations

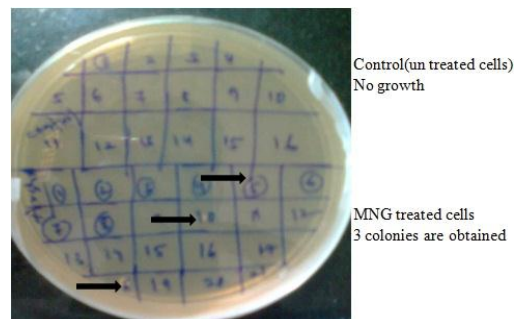


Figure 2: Isolation of Arginine hydroxamate (983 µg/ml) resistant colonies of *Corynebacterium glutamicum* by classical mutagenesis

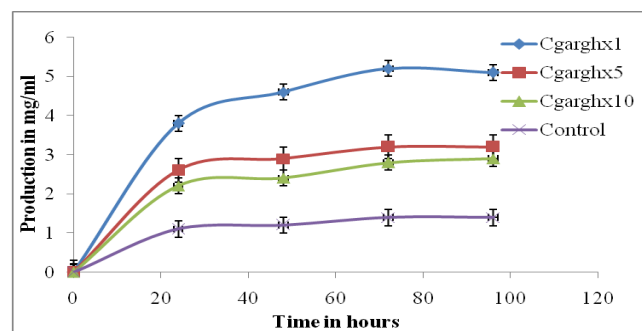


Figure 3: Arginine amino acid production from arginine hydroxamate resistant mutants and wild strain estimated by sakaguchi test