



IMPROVEMENT ON A MUTANT STRAIN OF *Corynebacterium glutamicum* FOR L-ISOLEUCINE PRODUCTION

*¹Subhadeep Ganguly and ²Ajit Kumar Banik

¹Assistant Professor, Department of Physiology, Vidyasagar College, Kolkata, West Bengal India.

²Former Professor and Head, Department of Chemical Engineering, Biochemical Engineering Sub-Division, Biotechnology Laboratory, University of Calcutta, 92, A. P. C. Road, Kolkata-700009.

***Corresponding Author: Dr. Subhadeep Ganguly**

Assistant Professor, Department of Physiology, Vidyasagar College, Kolkata, West Bengal India.

Article Received on 07/12/2017

Article Revised on 28/12/2017

Article Accepted on 18/01/2018

ABSTRACT

L-isoleucine is used as fuel by the muscle, sparing other L-amino acids from being burned up. Its economically viable production using fermentation was initially started with *Bacillus* sp and *Pseudomonas* sp from alpha amino butyric acid. In order to establish more economically advantageous procedure for this amino acid production, several trials have been made for last four decades. In present study, the authors intended to develop a new mutant of *Corynebacterium glutamicum* for L-isoleucine production. A high L-isoleucine yielding mutant *Corynebacterium glutamicum* X364 was developed from *Corynebacterium glutamicum* X153 by subsequent mutagenic treatments with EMS and UV irradiations respectively. *Corynebacterium glutamicum* X153 was first treated with EMS and total 49 mutants were isolated among which only 39 isolates accumulated L-isoleucine on subsequent fermentation trials. Further mutation with UV irradiations of the mutant *Corynebacterium glutamicum* X216 resulted development of total 36 new mutants among which *Corynebacterium glutamicum* X364 accumulated highest amount of (17.38 mg/ml) L-isoleucine in the fermentation broth.

[KEYWORDS: *Corynebacterium glutamicum*, EMS, UV irradiations, mutagenic treatments.].

INTRODUCTION

L-isoleucine is a branched chain essential amino acid required in diet as food and feed additives.^[1] It is also used in pharmaceutical and fine chemical industries. It is the most hydrophobic amino acid and exists as zwitterions in the solution.^[2] It helps to maintain the optimum blood sugar level and hepatic nutrient supply^[3] It exists as crystal forms^[4] The major demands (about 400 tons per year) for L-isoleucine were reported in USA, China, Europe, Japan, India and Southeast Asia.^[5] The amino acids are commonly produced either by chemical synthesis or protein hydrolysis.^[6] Recently, with the advancement of genetic engineering, the L-amino acid flux in *Corynebacterium glutamicum* has been gained the central position in the research of industrial microbiology.^[7-10]

Mutation has improved the productivity of the industrial microbiology.^[11] So, there are prospects of further increasing the L-isoleucine fermentation by following this rational approach of strain construction. Sahm *et al.* (1996) applied *Corynebacterium glutamicum* SM13 for L-isoleucine production¹. Gupta *et al.* (2012) developed an L-methionine high yielding strain *Micrococcus glutamicus* AB₂₀₀ by induced mutation which can accumulated up to 7.2 mg/ml of L-lysine in the broth.^[8,9]

Peng *et al.* (2015) applied *Brevibacterium flavum* KM011 for L-isoleucine fermentation.^[11]

Considering the facts, in this present investigation, we are intended to develop a high L-isoleucine yielding superior strain of *Corynebacterium glutamicum* by random induced mutation followed by selection.

MATERIALS AND METHODS

Microorganisms

Corynebacterium glutamicum X153, a biotin auxotrophic mutant was employed for this present investigation. The strain was maintained on nutrient-agar medium. Subculturing was carried out once in an every three weeks and the culture was stored at 4°C.^[12]

The following media were used

Complete Medium (CM)

Glucose, 1%; peptone, 0.5%; beef extract, 0.3%; yeast extract, 0.1% and pH, 7.0.^[12]

Medium for L-isoleucine production

Glucose, 10%; urea, 0.8%; K₂HPO₄, 0.1%; MgSO₄·7H₂O, 0.025%; yeast extract, 0.2% and pH, 7.0.^[12]

Minimal Medium (MM)

Glucose, 2%; urea, 0.2%; K₂HPO₄, 0.1%; MgSO₄.7H₂O, 0.025%; biotin, 0.5µg/ml and pH, 7.0. Solution of glucose was sterilized before mixing. This medium was used throughout the study.^[12]

Treatment with Ethyl Methane Sulfonate (EMS)

1 ml of cell suspension (containing 3X10³ cells) was added to 9 ml of EMS solution of different concentrations prepared from a stock solution having a concentration of 864 mM resulting in dilution of EMS to 117.54 mM, 66.28 mM and 21.66 mM. After a certain period of incubation (Table 1), 1 ml of sample was diluted in water in each case. The diluted microbial cells were then plated on CD agar medium and kept at 30°C for 2 days to develop discrete colonies^[12]

Treatment with UV irradiation

1 ml of bacterial cell suspension was taken in a petri dish (5 cm diameter) and exposed to UV rays, emitted from a 15 watt Hanovia germicidal lamp from a distance of 12 cm from different periods of exposure (Table 2). The treated cells were incubated at 28°C for 24h and selected colonies were transferred to yeast agar slant and stored at 4°C^[12]

Analysis of L-isoleucine

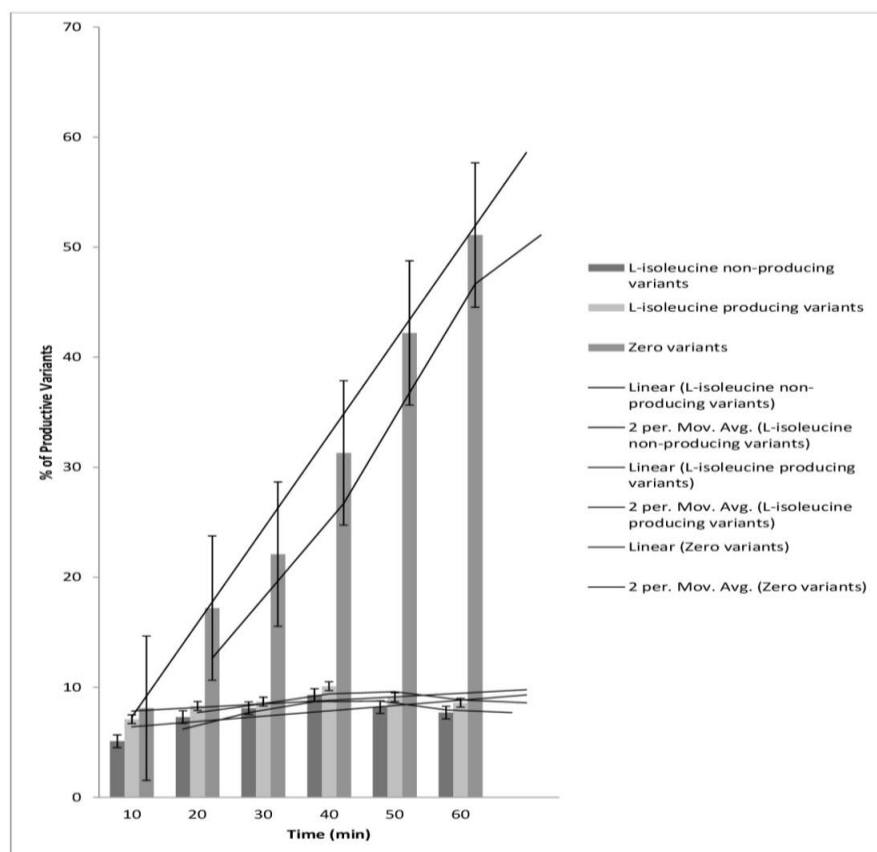
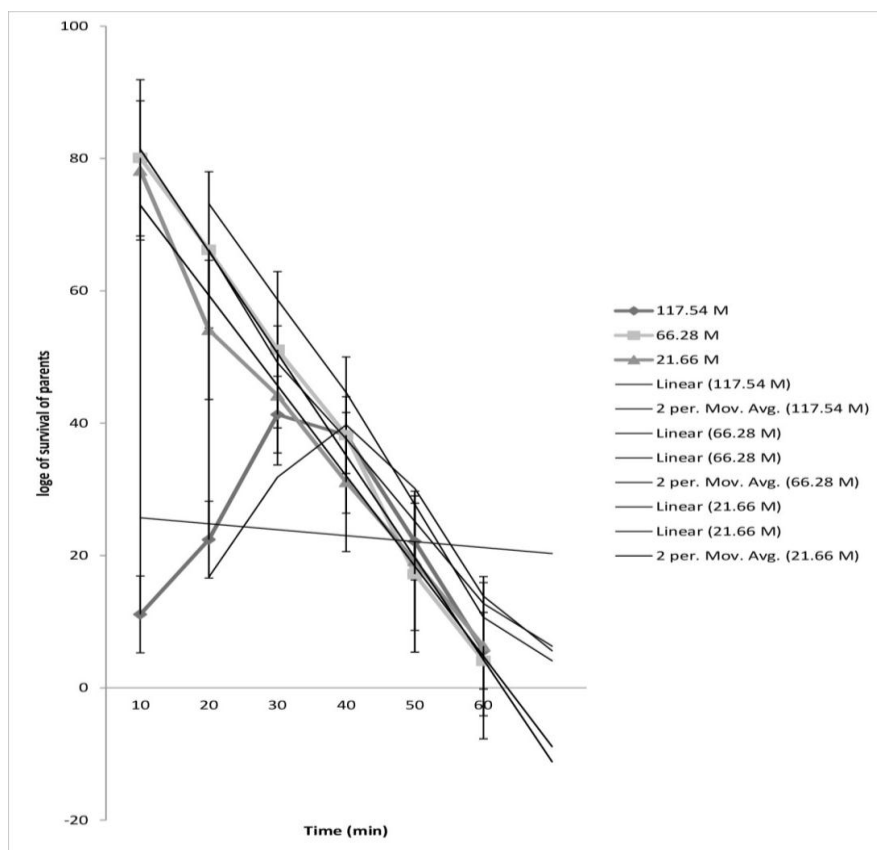
Descending paper chromatography was used for qualitative detection of L-isoleucine. Solvent system used includes n-butanol: acetic acid: water (2:1:1). The spots were visualized by spraying with a solution of 0.2% ninhydrin in acetone and quantitatively estimated using colorimetric estimation method.^[13]

Table 1: Relation between nutritional requirements of auxotrophic mutants obtained by the treatment of *Corynebacterium glutamicum* with EMS and their L-amino acid accumulation.

EMS (M)			117.54	66.28	21.66
Exposure time (min)			10	30	60
Total no of auxotrophs			21	11	17
Extracellular L-amino acid pattern		Similar	08	02	08
	L-isoleucine	More than	07	06	04
		Less than	02	-	02
	Other amino acids	L-leucine	01	-	01
		L-methionine	02	03	02
		L-threonine	01	-	-
		L-lysine	-	-	-
Requirements of nutrients		Yeast extract	17	07	04
		L-amino acids	13	05	08
		Vitamins	21	11	17
r			04	03	04
r = Frequency of spontaneous reversion X 10 ³					

Table 2: Relation between nutritional requirements of auxotrophic mutants obtained by exposing *Corynebacterium glutamicum* X181 to UV irradiations and their amino acid accumulation.

Periods of exposure to UV irradiations		15	30	45	60
Total no of auxotrophs		07	14	11	04
Extracellular L-isoleucine pattern	Similar to parents	01	03	02	02
	Less than parents	-	-	02	-
	More than parents	02	07	06	01
Other amino acid accumulation	L-leucine	01	01	01	01
	L-methionine	02	01	-	-
	L-threonine	-	02	-	-
	L-lysine	01	-	-	01
Requirements	Yeast extract	04	02	04	01
	L-amino acids	06	01	02	01
	Vitamins	07	14	11	04
r		02	02	01	03
r = Frequency of spontaneous reversion X 10 ³					



Captions/Legends for figures

1. Survival of *Corynebacterium glutamicum* obtained by the treatment with different dilutions of EMS (Values were expressed as mean $\pm$ SEM, where n=6)
2. Distribution of product variants obtained by EMS treatment (66.28M)

RESULTS AND DISCUSSION

Effect of EMS

A comparative study has been done on the survival of the bacterial strains (Fig.1). EMS concentration of 117.54 mM showed greater killing effect whereas 66.28 mM had moderate effect. The maximum development of the productive variants resulted when the period of treatment with EMS at a dilution 66.28 mM was 20 min giving survival % of 0.4 (Fig.2). Table 1 depicted the type of auxotrophs and their amino acid accumulation under EMS treatment. The parent organism accumulated only 1.31 mg/ml of L-isoleucine in the broth after 72h of incubation on a vertical rotary shaker shaking at a rate of 200 rpm. On exposure to EMS, the highest producer, *Corynebacterium glutamicum* X216 accumulated up to 6.83 mg/ml of L-isoleucine in the fermentation broth on subsequent fermentation trials.

The distribution of the productive variants of the EMS treatment has been depicted in Fig.2. The development of the auxotrophic high L-isoleucine producing mutants showed a maximum and then declined, but non L-isoleucine producing variants showed an increasing pattern even up to 60 minutes of the exposure. Thus, we have isolated 49 mutants after the treatment with different dilutions of EMS, which includes L-isoleucine producing variants, L-isoleucine non-producing variants and zero variants, among which only 39 isolates were tested for L-isoleucine production. *Corynebacterium glutamicum* X181 showed maximum L-isoleucine productivity (17 mg/ml). Table 1 showed different types of auxotrophic mutants of *Corynebacterium glutamicum* and their nutritional requirements developed by the treatments of different dilutions of EMS. The auxotrophic mutants showed varied pattern of L-isoleucine accumulation in the fermentation broth. A number of them (36.73%) maintained the pattern of parental accumulation. 34.69% showed similar to the parents and only 8.16% exhibited less accumulation of L-isoleucine compared to the parent strain. Four new amino acids, viz. L-leucine, L-methionine, L-threonine and L-lysine were found to be excreted by other auxotrophs. All the auxotrophs (49) isolated required vitamins for their growth. A constant rate of production was maintained in subsequent fermentation trials. Auxotrophs were classified according to their requirements of yeast extract, amino acids and vitamins.

Effect of UV irradiations

The bacterial suspension of *Corynebacterium glutamicum* X181 was then exposed to UV irradiations (15 watt) from a distance of 12 inches from a Hannovia germicidal lamp, for different periods of exposure (Table 2). The scoring of survival vs productive variants has been depicted in Fig.4. Complete inactivation of cells was resulted on exposure of 45 minutes. productive

variants were detected only upon the exposure of 30 minutes.

The mutational study revealed that, the mutagenic effect of EMS and UV irradiations on *Corynebacterium glutamicum* X216 has been well distinguished from nature of the survival curve. Both these two mutagens showed sharp killing effect.

Morbach *et al.* (1996) developed high L-isoleucine yielding strain *Corynebacterium glutamicum* SM13 by genetic engineering which can accumulate L-isoleucine up to 0.10 g per g of dry cell weight per hour in the fermentation broth.^[13] Ganguly and Banik used ethylenimine and UV irradiations for the development of high L-glutamic acid producing strain of *Micrococcus glutamicus*^[12] Gupta *et al.* (2012) developed L-lysine yielding strain *Micrococcus glutamicus* AB₂₀₀ from *Micrococcus glutamicus* AB₅ by induced mutation using UV rays and methyl methane sulfonate.^[14] Ganguly *et al.* (2014) used EMS and UV irradiations for the development of high L-methionine yielding strain of *Corynebacterium glutamicum*.^[15] Peng *et al.* (2015) used *Brevibacterium flavum* KM011 and reported accumulation of L-isoleucine up to 13.35 g/L.^[11] Ezemba *et al.* (2016) isolated a strain of *Microbacterium lacticum* from soil and tested its potential for the production of L-lysine in submerged fermentation using various hydrocarbons, sugars and nitrogen sources.^[16] Ma *et al.* (2016) conducted a study on over expression of *gnd*, *plg* and *fbp* in *Corynebacterium glutamicum* IWJ001 for improvement of L-isoleucine production^[17] Qingeng *et al.* (2017) employed metabolic engineering for development of a L-leucine producing recombinant *Corynebacterium glutamicum*^[18]

CONCLUSION

In this present study UV irradiations showed superiority over EMS treatments for the development of high L-isoleucine producing strain of *Corynebacterium glutamicum* X364 accumulated up to 17.38 mg/ml of L-threonine in the fermentation broth in the subsequent fermentation trials.

ACKNOWLEDGEMENT

We express our sincere gratitude to the PG Department of Botany of Utkal University and the Biochemical Engineering Division, Department of Chemical Engineering, University of Calcutta for their sincere cooperation without which we could not able to finish the work.

REFERENCES

1. Morbach S, Sahm H and Eggeling A, L-Isoleucine Production with *Corynebacterium glutamicum*: Further Flux Increase and Limitation of Export. *Appl Environ Microbiol*, 1996; 62: 4345.
2. Florence AT and Attwood D, Physiological principles of pharmacy, 4th edition, Pharmaceutical Press, London., 2006.
3. Kohlmeier, M. Isoleucine, In: Nutrient metabolism, Kohlmeier, M. (editor), Academic press, London, 2003; 377.

4. Xie XL, Wei M, Yunoki T, Kakehashi A, Yamano S, Kato M and Wanibuchi H, Long-term treatment with L-isoleucine and L-leucine in AIN-93G diet has promoting effects on rat bladder carcinogenesis. *Food Chem Toxicol*, 2012; 50: 3934.
5. Colon G E, Jetten MSM, Nguyen TT, Gubler ME, Follettie MT, Sinskey AJ and Stephanopoulos G, Effect of inducible *thrB* expression on amino acid production in *Corynebacterium lactofermentum* ATCC 21799. *Appl Environ Microbiol*, 1994; 61: 74.
6. Eggeling L, DeGraaf AA and Sahm H, Quantifying and directing metabolite flux: application to amino acid overproduction. *Adv Biochem Eng*, 1996; 54: 20.
7. Eikmanns BJ, Eggeling L and Sahm H, Molecular aspects of lysine, threonine, and isoleucine biosynthesis in *Corynebacterium glutamicum*. *Antonie van Leeuwenhoek*, 1994; 64: 145.
8. Dell KA, and Frost JW, Identification and removal of impediments to biocatalytic synthesis of aromatic acids from D-glucose: rate-limiting enzymes in the common pathway of aromatic amino acid biosynthesis. *J Am Chem Soc*, 1993; 115: 1581.
9. Morbach S, Kelle R, Winkels S, Sahm H and Eggeling L, Engineering the homoserine dehydrogenase and threonine dehydratase control points to analyse flux towards L-isoleucine in *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol*, 1996; 45: 612.
10. Morbach S, Sahm H and Eggeling L, Use of feedback-resistant threonine dehydratases of *Corynebacterium glutamicum* to increase carbon flux towards L-isoleucine. *Appl Environ Microbiol*, 1995; 61: 4315.
11. Peng LU, Lijun LIU and Wang J, The Optimization of L-Isoleucine Fermentation Conditions by *Brevibacterium flavum* KM011. *Enliven: Microbes and Microbial Techniques*, 1, 2015; 1.
12. Ganguly S, and Banik AK, Induced mutation and selection of High Yielding strain of *Micrococcus glutamicus* for glutamic acid production. *J Indian Chem Soc*, 2010; 87: 712.
13. Morbach S, Sahm H and Eggeling L, L-Isoleucine Production with *Corynebacterium glutamicum*: Further Flux Increase and Limitation of Export. *Appl and Environ Microbiol*, 1996; 62: 4345.
14. Gupta A, Das SK and Banik AK, Induced mutation and selection of a high yielding strain of *Micrococcus glutamicus* for l-lysine production. *Int J Pharm Bio Sci*, 2012; 3: B35
15. Ganguly S, Satapathy KB and Banik AK, Induced mutation, Development of Multiple Analogue Resistant Strain and Protoplast Fusion for L-methionine Fermentation. *Res Jof Pharmaceutical Dosage Forms Technol*, 2014; 6: 303.
16. Ezemba CC, Ozokpo CA, Anakwenze VN, Anaukwu GC, Ogbukagu CM, Ekwealor CC and Ekwealor IA, Lysine production of *Microbacterium lacticum* by submerged fermentation using various Hydrocarbon, sugar and Nitrogen sources. *Adv Microbiol*, 2016; 6: 797.
17. Ma W, Wang J, LiY, Shi F and Wang X, Enhancing pentose phosphate pathway in *Corynebacterium glutamicum* to improve L-isoleucine production, *Biotechnol Appl Biochem*, 2016; 63: 877.
18. Qingeng H, Ling L, Weibin W, Songgang W and Jianzhong H, Metabolic engineering of *Corynebacterium glutamicum* to enhance L-leucine production. *Afr J Biotechnol*, 2017; 16: 201.