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Isolation, characterization and improvement on a wild strain of *Corynebacterium glutamicum* for L-glutamic acid production

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L-Glutamic acid is a non-essential, proteinogenic amino acid, largely used as a flavoring agent, animal food additive and also in therapeutic purposes. Different strains of *Corynebacterium glutamicum* are widely used for its large scale production by means of fermentation. The present study was aimed to isolate and characterize an wild strain of *Corynebacterium glutamicum* from North Bengal soil and then to develop high L-glutamic acid yielding mutant strain from it by subjecting it to subsequent random mutagenic treatments using UV irradiation and EMS respectively. In this course of study, a wild strain of *Corynebacterium glutamicum* was isolated from the collected soil which could produce up to 1.8 mg/ml L-glutamic acid in the fermentation broth on subsequent fermentation trials. This strain was subjected to mutagenic exposure for random mutation using UV irradiations and nitrous acid. In this study, nitrous acid gained superiority in the context of the development of high L-glutamic acid producing mutant. *Corynebacterium glutamicum* X680 was developed as a superior most strain in this experimental trial which could accumulate up to 7.4 mg/ml L-glutamic acid in the fermentation broth.

Keywords: Isolate, characterize, wild strain, mutant, *Corynebacterium glutamicum*, UV irradiation, nitrous acid.

Introduction

L-Glutamic acid, a non-essential proteinogenc amino acid, was the first amino acid produced in large scale by fermentation since the discovery of *Corynebacterium glutmicum* from soil by Kinoshita *et al.*[1]. This microbe is a rod shaped, non-sporulating, Gram-positive, non-pathogenic, biotin auxotrophic bacterium which contains mycolic acids and widely distributed in the nature. The taste of L-glutamic acid is neither sweet, sour, salty nor bitter and is known as 'umami'; its sodium salt known as monosodium glutamate (MSG) is widely used as a popular flavoring agent[2]. It is also used as animal food additive and therapeutic agent[3]. US-FDA declared MSG as 'generally recognized as safe' (GRAS) as food additive, favors its market expansion. Due to its huge demand, its market is expected to experience a considerable growth. Its global market was estimated over 2.9 million in 2014 and is expected to reach more than 4 million tons by 2023 (A report published by Global Market Insights, Report ID: GMI225). Asian pacific was estimated as the highest consumer of L-glutamic acid in the globe. Japan, Taiwan, South Korea, China and India are the major Asian L-glutamic acid consumer countries. America, Middle East countries and also African countries consume a large volume of L-glutamic acid and its products. China, Thiland, Indonesia, Taiwan and Vietnam are the major L-glutamic acid producer countries, among which China accounted to more than 65% of its global L-glutamic acid and its monosodium salt producer. It was discovered by German chemist Kerl Heinrich Ritthausen in 1866[4]. But its large scale production was started in the first half of 20th century by Ajinomoto co in Japan using chemical synthesis method[5]. However, after the discovery of L-glutamic acid producing strain *Corynebacterium glutmicum*, its production by fermentation gained advantage over chemical synthesis as the former produced sterio-specific L-isomer, where as the later yielded racemic mixture[5].

Since then several strains of *Corynebacterium glutmicum* were tested for large scale production of L-glutamic acid using submerged fermentation[6]. With the advancement of biotechnology and genetic engineering, it was further possible to develop genetically modified mutants of *Corynebacterium glutmicum* for over production of L-glutamic acid[7–11].

In this present study, the authors were intended to isolate an L-glutamic acid producing wild strain of *Corynebacterium glutamicum* from North Bengal soil and subsequently

to develop a superior mutant strain from this wild strain by subsequent random mutational treatments using nitrous acid and UV irradiations.

Materials and methods:

Isolation and characterization of Corynebcterium glutamicum:

Collection of soil sample: Soil samples were collected from five distinctive areas of North Bengal, West Bengal, India. Each sample was packed properly in a clean polythene bag, labeled and then carried to our laboratory for analysis.

Isolation of Corynebcterium glutamicum: A weighted amount of 10 g each soil sample was separately added to 100 ml deionized, double distilled water in a 250 ml Erlenmeyer conical flask. A ten-fold serial dilution of the soil suspension was made in each case using deionized, double distilled water. A loopful (0.1 ml) sample from each 1:100 diluted soil suspension was inoculated onto petridish (5 cm diameter) containing nutrient-agar medium and allowed to grow for 48 h at 30°C. Discrete, well isolated colonies were developed. They were subcultured using slants containing nutrient-agar medium. The isolates were examined for identification and production of L-glutamic acid.

Characterization of the colonies:

Morphological characterization: All the incubated media containing inoculum, after 48 h of incubation at 30°C in an incubator were tested for characterization of different colonies like forms, elevation, clusters, size, pigmentation, mergin etc. Morphological characterization includes cell shape, arrangements and staining properties including Gramstaining, spore staining and acid fast staining[12].

Biochemical characterization: The bacterial colonies showing higher L-glutamic acid productivity than parent strain was subjected to various routine biochemical analysis (like methyl red test, Voges proskaur test; utilization of carbohydrates like glucose, fructose, xylose, arabinose, ribise, mannitol, maltose, sucrose, lactose, dextrin starch etc; antibiotic tests like amphicilin, streptomycin and chloramphenicol tests; utilization and reduction of nitrate; urease test)[13].

Composition of growth medium: The bacterial growth medium was composed of: glucose, 2%; peptone, 0.5%; yeast extract, 0.1%; beef extract, 0.3%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; $MgSO_4.H_2O$, 0.025%; agar, 4% and H_2O, 1 L[14].

Composition minimal salt medium for L-glutamic acid production: L-Glutamic acid production was carried out using basal salt medium containing: glucose, 10%; urea, 0.8%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; $MgSO_4.H_2O$, 0.025%; yeast extract, 0.2% and H_2O, 1 L[15]. The pH was adjusted at 7.0. The submerged fermentation was carried out at 30°C for 72 h.

Analytical method for L-glutamic acid estimation: Descending paper chromatography was employed for qualitative detection of L-glutamic acid in the broth. The chromatographic run was conducted for 16 h using a solvent system include n-butanol:acetic acid:water (2:1:1) and then spots were visualized by spraying 0.2% ninhydrin in acetone. The quantitative estimation was done by colorimetric estimation method[16,17].

Mutagenic treatments:

Treatment with Ethyl Methane Sulfonate (EMS): Cell suspension (1 ml containing 10^6 cells) was added to 9 ml EMS solution of different dilutions (180 mmol/ml, 120 mmol/ml and 60 mmol/ml) prepared from a stock (500 mmol/ml). After 30 min incubation, 1 ml cell suspension was diluted by adding 9 ml distilled water. The diluted cell suspension was plated in agar nutrient medium and allowed for 48 h for the development of discrete colonies in ease case[15].

Treatment with UV irradiation: Bacterial suspension (2 ml) containing 10^6 cells/ml was taken in a petridish (5 cm diameter) and exposed to 300 erg/mm^2 from a distance of 12 cm from different time intervals (5–60 s). The source of radiation was Hannovia germicidal lamp (model 26501NDL3X). The treated cells were incubated at 30°C for 48 h and selected colonies were transferred to nutrient-agar slant and stored at 4°C[15].

Results and discussion

Mutation has gained a lot of advantage in improving the productivity of bio-based industries[18,19]. Strain improvement plays a vital role in the commercial development of amino acid fermentation. Classical mutagenesis followed by random selection is a reliable short term strain development method which is frequently used as a cost effective method of choice[20]. The most common method generally adopted to get high yielding mutants is to treat the microbial cells with

mutagenic agents until a certain 'desired' kill is resulted. The survivors are allowed to grow in plates to form colony or a randomly selected group of colonies for product formation in submerged fermentation[21]. The most commonly used mutagens include ethylmethane sulfonate (EMS), nitrous acid, nitrosoguanidine (NTG), 4-nitroquinolone-1-oxide, gama rays, UV irradiation etc. The optimum level of killing for improved metabolite productivity is thought to be in the range of 70–99.99%[22]. Almost nothing is known about the mechanism of improved production by high yielding mutants. Initially, scientists thought that the end product of a primary metabolite exerts strict control over the accumulation of an intermediate by an auxotrophic mutant. At a growth limiting concentration of the end product would result a large accumulation of the substrate due to deficiency of specific enzyme. Due to such decrease in concentration of the repressive end product, the feed-back inhibition or repression is by passed which is prominently exhibited by the auxotrophs. Indeed, auxotrophic mutation has been a crucial factor in the production of primary metabolites including amino acids in large scale. When multiple primary metabolites are produced by a single (multi-branched) pathway, mutation of one branch often led to over production of other branch.

Treatment with EMS: A comparative study has been conducted on the survival of the wild strin *Corynebacterium glutamicum* X60, isolated from North Bengal soil. Fig. 1a exhibited 180 mmol/ml EMS had the greater killing effect, where as 120 mmol/ml showed moderate killing effect. The maximum productive variants were obtained with 60 min exposure of 60 mmol/ml EMS with a survival of 0.8% (Fig. 1b).

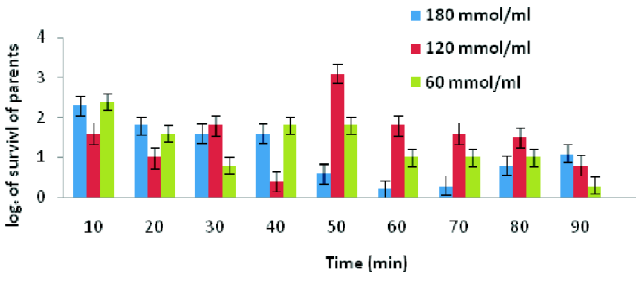

Fig. 1a. Survival in terms of loge of *Corynebacterium glutamicum* X60 obtained by exposure of different concentrations of EMS for different time intervals (Values were expressed as mean±SEM, where n = 6).

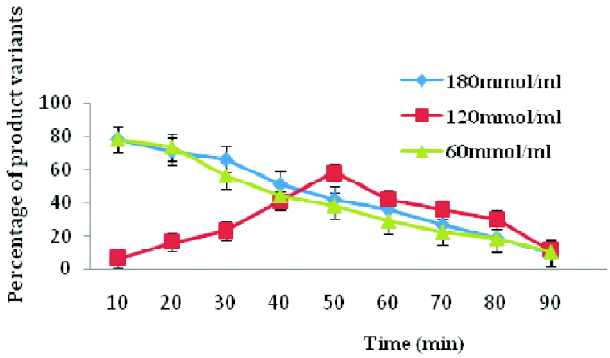

Fig. 1b. Survival (%) of *Corynebacterium glutamicum* X60 obtained by exposure of different concentrations of EMS for different time intervals (Values were expressed as men±SEM, where n = 6).

The distribution of productive variants of the EMS treatment is presented in Fig. 2. Both the zero variants and L-glutamic acid productive variants reached maximum on 50 min of exposure and then declined sharply, where as non-L-glutamic acid productive variants showed increasing pattern even up to 90 min of exposure in 120 mmol/ml EMS (Fig. 2).

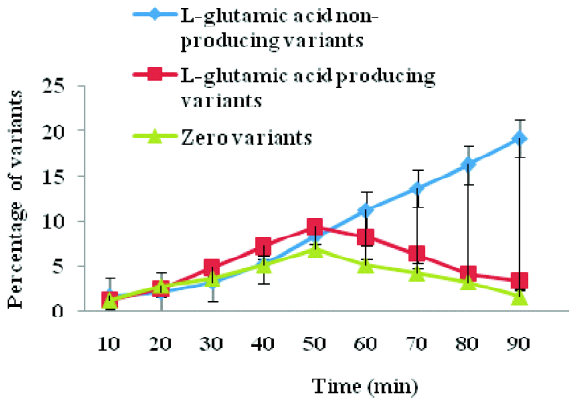

Fig. 2. Distribution of productive variants obtained by EMS (120 mmol/ml) treatment (Values were expressed as mean ±SEM, where n = 6).

In this course of treatment, 319 different mutant strains were isolated, out of which 45 mutants accumulated higher amount of L-glutamic acid than parent strain (Table 1). *Corynebacterium glutamicum* X176 produced maximum amount of L-glutamic acid in subsequent fermentation trials (4.2 mg/ml).

Table 1. Nutritional requirements and amino acid production by the auxotrophic mutants developed by EMS treatments

EMS mmol/ml	Exposure time (min)	Total No. of auxotrophs isolated	Extracellular L-glutamic acid pattern (compare to parents)			Other amino acids production			Nutritional requirements			*r*
			Similar	Less than	More than	L-Lysine	L-Methionine	L-Threonine	Yeast extract	Vitamins	Amino acids	
180	10	06	–	–	02	02	01	01	03	06	02	01
	20	11	04	05	–	01	–	01	04	11	–	01
	30	04	–	–	02	02	–	–	02	04	01	03
	40	04	–	–	04	–	–	–	01	04	01	01
	50	08	02	02	–	01	01	02	01	08	03	02
	60	02	–	–	02	–	–	–	–	02	–	01
	70	07	02	02	–	02	–	01	03	07	02	01
	80	04	–	01	01	–	01	01	01	04	01	02
	90	03	01	02	–	–	–	–	–	04	–	02
120	10	04	01	–	–	–	02	01	01	04	02	01
	20	07	–	–	03	–	02	02	–	06	05	01
	30	11	–	–	–	–	07	02	02	07	03	01
	40	17	04	02	06	–	03	02	04	11	06	01
	50	08	02	02	–	02	–	02	02	04	06	02
	60	16	06	03	02	–	02	03	–	11	04	01
	70	06	04	–	02	–	–	–	–	06	03	01
	80	06	02	–	–	–	02	02	–	04	02	01
	90	11	06	02	03	–	–	–	–	07	02	01
60	10	18	07	02	01	–	02	06	–	11	04	02
	20	22	07	04	–	02	03	06	01	17	05	02
	30	24	10	06	04	–	–	04	04	08	07	01
	40	21	09	07	02	–	–	03	–	14	06	01
	50	17	07	07	–	–	02	01	–	13	06	01
	60	21	07	03	05	–	02	04	–	14	–	01
	70	16	04	03	03	–	03	01	02	04	11	01
	80	24	11	07	01	–	03	02	–	14	06	01
	90	21	05	04	02	–	05	04	01	18	07	01

r, frequency of spontaneous reversion×10^3.

Treatment with UV irradiation: A superior mutant *Corynebacterium glutamicum* X176 for L-glutamic acid production developed by EMS treatment was then subjected to further mutational induction using UV irradiation to scale up the process. Bacterial suspension was then exposed to 300 erg/mm^2 UV irradiation from a distance of 12 cm using Hanovia germicidal lamp for different time intervals (5–60 s). The scoring of survivors and productive variants were presented in Fig. 3.

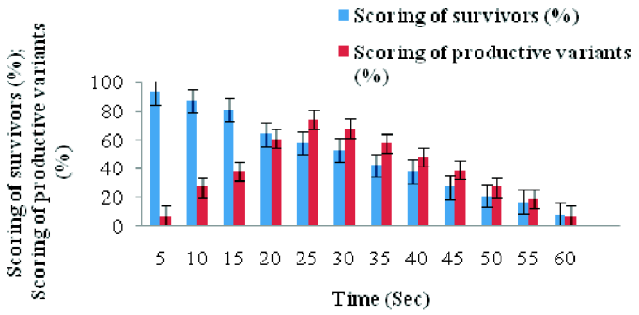

Fig. 3. The scoring of survivor and productive variants obtained by treatment of *Corynebacterium glutamicum* X176 by UV irradiation (Values were expressed sa mean±SEM, where n = 6).

Almost complete inactivation took place on 60 s of exposure. Maximum productive variants were obtained on 25 s of exposure, and then the number decreased gradually. A maximum 34 productive variants were obtained in this present study on exposure of UV irradiation for 25 s (Table 2). *Corynebacterium glutamicum* X680 appeared as highest L-glutamic acid producer (8.2 mg/ml).

Ganguly and Banik (2010) developed a glutamic acid producing strain of *Micrococcus glutamicus* by induced mutation using UV irradiation[15]. Pasha *et al.* (2011) used UV irradiation and nitrosoguanidine as effective mutagens for the development of high L-glutamic acid yielding strain of *Corynebacterium glutamicum*[23]. *Bacillus subtilis* OUT8103 was subjected to classical mutagenesis for the development of high yielding strain for L-arginine production[24]. Induced mutation using ethylmethane sulfonate and UV irradiation appeared to be very effective tool for the development of high L-methionine yielding strain of *Corynebacterium glutamicum*[25]. Very recently, Reddy *et al.* (2017) aimed to develop a L-glutamic acid overproducing mutant strain *Corynebacterium glutamicum* K051 from an wild strain iso-

Table 2. Induction of mutagenic treatment with UV irradiation for scale up L-glutamic acid production using a mutant *Corynebacterium glutamicum* X176

Periods of exposure to UV irradiation (s)	Total No. of auxotrophs isolated	Extracellular amino acid accumulation pattern										
		L-Glutamic acid (compare to parents strain)			Other amino acids			Nutritional requirements				
		Similar	More than	Less than	L-Lysine	L-Methionine	L-Threonine	Yeast extract	Vitamins	Amino acids	*r*
05	16	01	03	06	03	02	01	04	16	07	01
10	08	04	01	02	–	01	–	05	06	02	01
15	11	06	01	02	–	02	–	04	07	03	03
20	17	06	04	02	–	02	03	04	11	05	01
25	17	03	11	03	–	–	–	–	15	05	01
30	05	01	02	–	–	–	02	02	04	03	01
35	21	11	02	07	–	–	01	–	17	–	02
40	11	04	03	04	–	–	–	–	11	02	01
45	21	04	02	06	02	04	03	07	05	15	01
50	17	03	01	11	02	–	–	–	11	15	03
55	07	03	02	02	–	–	–	06	–	05	01
60	09	02	02	04	–	–	01	02	07	02	01

r, frequency of spontaneous reversion×10^3.

lated from sewage contaminated soil of Anantapuramu by induced mutation using UV irradition[26].

Conclusion

In this present study UV irradiation appeared to effective mutagen for the development of high L-glutamic yielding mutant *Corynebacterium glutamicum* X680 from *Corynebacterium glutamicum* X60, which could accumulated up to 7.4 mg/ml L-glutamic acid in subsequent fermentation trials. However, further optimization of different physico-chemical parameters is needed to further scale up the process.

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References

1. S. Y. Pasha, M. N. Ali H. Tabassum and M. K. Mohd, *Int. J. Engg. Sci. Technol.,* 2015, **3**, 3941.
2. J. Nakamura S. Hirano, H. Ito and M. Wachi, *Appl. Environ. Microbiol*., 2007, **73**, 4491.
3. S. Dutta, S. Ray and K. Nagrajan, *Saudi. Pharm. J.,* 2013, **21**, 337.
4. R. Shyamkumar, I. M. Moothy, K. Ponmuugan and R. Basak, *Avicenna J. Med. Biotechnol*., 2014, **6**, 163.
5. J. Birnbaum and A. L. Demain, *Appl. Microbiol*., 1969, **18**, 287.
6. T. Hermann, *J. Biotechnol*., 2003, **104**, 155.
7. Yu Wang, G. Cao, Xu Deyu, F. Liwen, X. Wu, X. Ni, S. Zhao, P. Zheng, J. Sun and Y. Ma, *Appl. Environ. Microbiol.,* 2018, **84**, 1.
8. C. V. K. Reddy, U. Keerthi, A. V. N. Swamy and D. M. Rao, *Biosci. Biotechnol. Res. Asia*, 2017, **14**, 415.
9. D. Lubitz and F. Wendisch, *BMC Microbiol*., 2016, **16**, 2.
10. S. Nadeem, B. Niaz, H. M. Muzammil, S. M. Rana, S. M. Rajoka and A. R. Shkoori, *Pakistan J. Zool.,* 2011, **43**, 285.
11. N. S. Khan and B. Prasad, *Biochem. Engg. J.,* 2005, **25**, 173.
12. B. Musa, S. A. Ado and I. O. Abdullahi, *American Journal of Microbiology and Immunology,* 2016, **1**, 16.
13. B. Blombach and G. M. Seibold, *Appl. Microbiol. Biotechnol*., 2010, **86(5)**, 1313.
14. S. Ganguly and A. K. Banik, *J. Indian Chem. Soc*., 2011, **88**, 707.
15. S. Ganguly and A. K. Banik, *J. Indian Chem. Soc.,* 2010, **87**, 717.
16. K. M. Nampoothiri and A. Pandey, *Biotechnol. Lett*., 1996, **18**, 199.
17. S. Ganguly and A. K. Banik, *Biosci. Biotechnol. Res. Asia*, 2011, **8**, 717.
18. V. A. Vinci and G. Byng, "Strain improvement by nonrecombinant methods. Manual of Industrial Microbiology and Biotechnology", 2nd ed., eds. A. L. Demain and J. E. Davies, ASM Press, Washington, DC, 1999, pp. 103.
19. V. A. Vinci, T. D. Hoerner, A. D. Coffman, T. G. Schimmel, R. L. Dabora, A. C. Kirpekar, C. L. Ruby and R. W. Stieber, *J. Indust. Microbiol*., 1991, **8**, 113.
20. T. Rowlans*, Microbiol. Technol.,* 1984, **6**, 3.
21. L. Jose, Adrio and A. L. Demain, *FEMS Microbiol. Rev*., 2006, **30**, 187.
22. I. N. Simpson and C. E. Caten, *J. Gen. Microbiol*., 1979, **110**, 1.
23. S. Y. Pasha, M. N. Ali, H. Tabassum and M. Mohd. Khan, *International Journal of Engineering Science and Technology*, 2011, **3**, 3941.
24. H. R. V. N. G. Rao, H. N. Shravani, S. S. Vutukuru, K. Subramanyam and P. Rajasekhar, *International Journal of Science and Technology*, 2012, **2**, 16.
25. S. Ganguly, K. B. Satapathy and A. K. Banik, *Research Journal of Pharmaceutical Dose Forms and Technology*, 2014, **6**, 303.
26. C. V. K. Reddy, A. V. N. S. Keerthi and D. M. Rao, *Biosci. Biotechnol. Res. Asia,* 2017, **14**, 415.