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Improvement of Wild Bacterial Strain NIAB-SM-3 for Better Lysine Production Using N-methyl-N′-nitro-N-nitrosoguanidine (NTG)

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Abstract.- Classical chemical mutagenesis is one of the important tools in improving the bacterial strains for the production of desired metabolites. The N-methyl-N′-nitro-N-nitrosoguanidine (NTG) is one of the commonly employed mutagen for the improvement of bacterial strain for better amino acid production. In the present research, wild strain NIAB-SM-3 was isolated from molasses dumped soil. It produced 3.0g/L of lysine along with glutamic acid and alanine when grown in molasses based medium. This wild strain was mutated with the help of NTG. Minimum inhibitory concentrations (MIC) for NTG and lysine analogue, S-(β-aminoethyl)-L-cysteine (AEC), were found to be 50µg/ml and 625µg/ml, respectively. On the basis of AEC resistance 43 out of total 57 mutants were selected and tested on different media for their abilities to produce lysine in fermentation broth. Among the molasses media, NIAB-NT-43 gave maximum lysine production *i.e.* 3.8g/L in M-I medium. On the basis of their lysine production in molasses media, 16 mutants were selected and cultured in glucose medium, L-6. Almost all of them produced lysine but maximum production of 5.0g/L was observed by NIAB-NT-15. In the nutshell, two-fold increase in lysine was observed as a result of chemical mutagenesis through the use of NTG. Furthermore, repeated classical chemical mutagenesis and tools of genetic engineering can be employed in the future to enhance lysine production.

Key words: Amino acid production, chemical mutagenesis, lysine, AEC analogues, molasses.

INTRODUCTION

Amino acids play a crucial role in virtually all biological processes and are provided by protein in the diet. The significance and remarkable scope of their functions are catalysis, transport, coordinated motion, mechanical support and transmission of nerve impulse and control of growth (Park and Lee, 2008). Diets that contain sufficient carbohydrates and other essential components could be deficient in one or more of the required amino acids, such as wheat proteins are low in lysine and tryptophan (Anjum *et al*., 2005; Bankar and Singhal, 2011).

Lysine is an essential amino acid for both humans and animals. It is usually the first or second limiting amino acid in majority of the cereal based formulation (Nadeem *et al*., 1996; Zabala-Diaz, 2003) and is one of the most important amino acids in forage addition as well as in animal nutrition (Seep-Feldhaus *et al*., 1991; Schrumpf *et al*., 1991). Several experiments demonstrated a linear response in early body weight gain to increasing concentrations of balanced dietary lysine. Importance of lysine also lies in regulation of different pathways with its active role in enzyme modulation (Lemme, 2003; Quentin *et al*., 2005).

Micro-organisms have been used commercially for over 40 years to produce amino acids (Eggeling and Sahm, 1999; Shakoori *et al*., 2012). It is more advantageous to use wild type organism (Bröer and Kramer, 1991; Ali *et al*., 2011). An organism will produce sufficient amount of different amino acids during normal growth, to meet its needs for protein synthesis (Clarke, 1988). Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes (Ikeda, 2003; Mitsuhashi, 2014). Classical chemical mutagenesis, optimization of fermentation conditions and tools of genetic engineering are in

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use for the improvement of bacterial strains for better lysine production (Becker and Wittmann, 2012). In the present project successful efforts were made to improve the wild strain of *Escherichia coli* NIAB-SM-3 isolated from molasses dumped soil for better lysine production.

MATERIALS AND METHODS

Wild strain NIAB-SM-3 used in this research was isolated earlier from molasses-rich soil near Crescent Sugar Mills, Faisalabad. Materials and glassware were procured from local market, while reagents (analytical and HPLC grade) and standards were purchased from Sigma-Aldrich (Tokyo, Japan) and Merck (Darmstadt, Germany).

Preparation of media

Three molasses based media, M-I, M-II and M-III, and one glucose-trypticase medium, L-6 (modified according to local conditions) were used. Fermentation media contains the following composition (in g l^{-1}) molasses, 100g; KH_2PO_4, 0.5g; $K_2HPO_4.3H_2O$, 0.5g and $MgSO_4.7H_2O$ (0.25g). 20 g of $(NH_4)_2SO_4$, NH_4NO_3 and NH_4Cl added as ammonium salts in M-I, M-II and M-III, respectively. In all these media $CaCO_3$ (20g) was used as the buffering agent. Media were sterilized through autoclaving at 121°C and 15lbs pressure for 15 min. Moreover, one glucose-trypticase medium L-6 was also used with some modification. A minimal medium MM-12 was also employed for the mutation study.

Minimum inhibitory concentration (MIC)

MIC of NTG and lysine analogue, S-(β-aminoethyl)-L-cysteine (AEC), against the wild type cells was worked out following the method of Nadeem *et al*. (2001) with slight modification. A minimal medium MM-12 was selected as seeding layer and the same with 2% agar was used as the basal medium as well. Different concentrations of NTG (25-250 µg/ml) and AEC (625-5000µg/ml) were prepared in 0.1M phosphate buffer (pH, 7.0). Bioassay cup or rings were filled with 0.8ml solution of prepared concentrations and incubated up to 48 hours at 28°C. Thereafter, the inhibition zones diameters were measured, and MIC was calculated.

Classical chemical mutagenesis

Improvement in lysine production by the bacterial isolates was done through chemical mutation, using NTG that causes deregulation in the biosynthetic pathway of the organisms. Bacterial isolates were grown on nutrient broth and its nephalometric value was noted. Dilutions were made to get the population density of $2.0 \times 10^{8;}$ this culture was treated with NTG @ 50µg/ml to induce mutagenesis.

Isolation of AEC-resistant mutants

Three batches of Petri plates containing different media were prepared *i.e*. Simple nutrient agar medium (B-I), minimal medium + agar (B-II), and minimal medium + agar + AEC solution (B-III). The NTG treated culture was incubated in these Petri plates and at 28°C for 1-2 days. The mutants appearing in B-III were AEC-resistant mutants.

Fermentation

The fermentation broth was prepared using different media and their composition is already mentioned (Nadeem *et al*., 2011). For the purpose, growth of bacterial isolates were maintained in 250ml Erlenmeyer flasks at 30±1°C and 150 rpm in a specialized gyratory shaker for a maximum of 96 hours. Afterwards, growth was terminated and harvest pH noted. Samples were made cell free by centrifugation at 2800 rpm for 10 min. The supernatant was filtered for complete purity.

Amino acid analysis

Amino acids were analyzed as ortho-phthaldehyde derivatives by reverse phase chromatography using an AminoQuant column with a Hewlett Packard series 1050 high-pressure liquid chromatography (HPLC) system (Colon *et al*., 1995).

RESULTS AND DISCUSSION

Conventionally, the strain improvement has been achieved through mutation, selection, or genetic recombination. Overproduction of primary or secondary metabolites is a complex process, and successful development of improved strains requires knowledge of physiology, pathway regulation and

control, and the design of creative screening procedures. In addition, it also requires competence of the fermentation process for each new strain, as well as sound engineering know-how for media optimization and the fine-tuning of process conditions (Parekh *et al*., 2000). Wild type NIAB-SM-3 was selected for improvement. When grown in M-III, it produced 3.0g/L of lysine in M-III and 2.4g/L of lysine in M-II and only 0.93g/L of lysine in M-I medium. It also produced alanine and glutamic acid in some quantities. The wild strains usually unable to produce amino acids in appreciable quantities. Therefore, classical mutagenesis is employed for the hyper-production of various metabolites. In this current study, the NTG was used at varying concentration. The survival percentages were tabulated indicating that nearly 3-4% of the cells survived when exposed to a dose of 50µg/ml for a period of 90 minutes (Fig. 1). In the second phase, minimum inhibitory concentrations (MIC) were chalked out for NTG and AEC *i.e.* 50µg/ml and 625µg/ml, respectively (Figs. 2, 3).

The culture treated with NTG was incubated and 57 mutants were isolated and growth was maintained on nutrient agar. In order to isolate AEC resistant, mutants were incubated on B-III and amongst, 43 mutants were found AEC resistant. The analogue resistant mutant isolation is important to obtain such mutants that are insensitive to feedback inhibition or repression, and AEC resistant mutants are of such kind. The amino acid resistance may be due to increased transcription or translation of a gene that will reduce, in some case, allosteric effect of the amino acid analogue. But the resistance to AEC does not necessarily mean the lysine production. In addition, increasing the metabolic flux through the pathway by blocking branch pathway increases the level of end product of the pathway (Sen, 1991).

While tested in molasses media, M-I, M-II and M-III, eight out of 43 wild type isolates produced lysine in a range of 2.2-3.4g/l in M-I, nine produced lysine in M-II in a range of 2.0-3.2 g/l, whereas medium M-III proved to be the least supportive for lysine production and only two isolate produced 1.5-2.0g/l lysine in the fermentation broth (Table I).

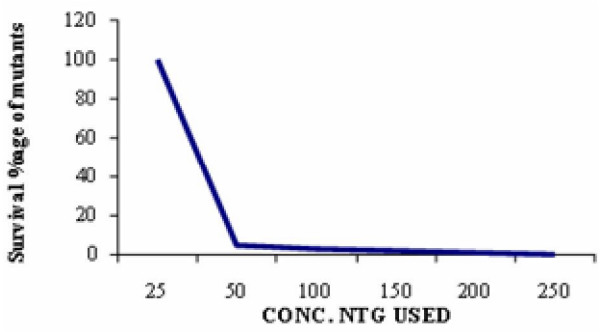

Fig. 1. Survival of mutants at various doses of NTG.

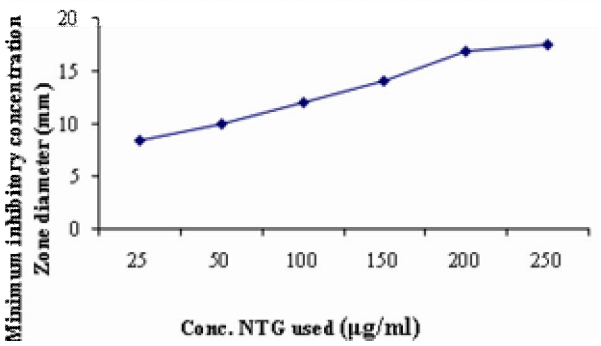

Fig. 2. Minimum inhibitory concentration (mm) at various concentrations of NTG

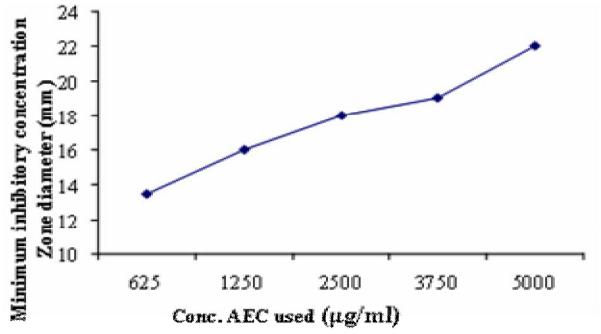

Fig. 3. Minimum inhibitory concentration (mm) at various concentrations of AEC.

Mutants showed slight increase in the production level. However, the behavior of mutants varied according to the media used. Among the molasses media, best results were produced by NIAB-NT-42 in M-I with a maximum of 3.8 g/L of lysine followed by 3.40g/L by NIAB-NT-43 in the same medium. The concentrations of different nutrients usually affect the production of amino

acids. The similar trends have been reported by other researchers too *e.g.* Bajwa *et al*. (2010) and Ali *et al*. (2009).

Table I.- Time scale production of lysine in different fermentation media.

Mutants	Molasses based Medium M-I	Molasses based Medium M-II	Molasses based Medium M-III
NIAB-NT-1	---**	1.36$_{(72hrs)}$	0.70$_{(72hrs)}$
NIAB-NT-2	---**	2.10$_{(72hrs)}$	0.20$_{(72hrs)}$
NIAB-NT-3	1.50$_{(96hrs)}$	0.75$_{(24hrs)}$	0.30$_{(72hrs)}$
NIAB-NT-4	1.10$_{(24hrs)}$	1.19$_{(24hrs)}$	1.50$_{(72hrs)}$
NIAB-NT-5	0.20$_{(24hrs)}$	1.46$_{(24hrs)}$	0.50$_{(96hrs)}$
NIAB-NT-6	0.90$_{(24hrs)}$	1.17$_{(24hrs)}$	0.60$_{(24hrs)}$
NIAB-NT-7	0.90$_{(24hrs)}$	1.36$_{(72hrs)}$	0.50$_{(96hrs)}$
NIAB-NT-8	1.60$_{(24hrs)}$	2.62$_{(24hrs)}$	0.60$_{(96hrs)}$
NIAB-NT-9	1.30$_{(48hrs)}$	1.96$_{(72hrs)}$	0.20$_{(96hrs)}$
NIAB-NT-10	1.40$_{(24hrs)}$	---**	0.50$_{(72hrs)}$
NIAB-NT-11	0.90$_{(24hrs)}$	1.34$_{(72hrs)}$	0.80$_{(96hrs)}$
NIAB-NT-12	0.70$_{(24hrs)}$	2.03$_{(96hrs)}$	1.10$_{(72hrs)}$
NIAB-NT-13	2.20$_{(24hrs)}$	1.86$_{(72hrs)}$	0.40$_{(96hrs)}$
NIAB-NT-14	1.80$_{(24hrs)}$	1.40$_{(72hrs)}$	0.80$_{(72hrs)}$
NIAB-NT-15	2.40$_{(72hrs)}$	1.60$_{(72hrs)}$	---**
NIAB-NT-16	2.70$_{(72hrs)}$	1.72$_{(24hrs)}$	0.80$_{(48hrs)}$
NIAB-NT-17	0.80$_{(24hrs)}$	1.56$_{(72hrs)}$	0.30$_{(96hrs)}$
NIAB-NT-18	2.30$_{(72hrs)}$	1.80$_{(72hrs)}$	0.50$_{(96hrs)}$
NIAB-NT-19	1.80$_{(72hrs)}$	1.14$_{(72hrs)}$	0.50$_{(96hrs)}$
NIAB-NT-20	0.10$_{(24hrs)}$	1.81$_{(72hrs)}$	0.10$_{(72hrs)}$
NIAB-NT-21	0.90$_{(24hrs)}$	2.02$_{(96hrs)}$	0.90$_{(24hrs)}$
NIAB-NT-22	0.20$_{(24hrs)}$	1.14$_{(72hrs)}$	0.20$_{(48hrs)}$
NIAB-NT-23	0.90$_{(72hrs)}$	2.14$_{(72hrs)}$	0.30$_{(24hrs)}$
NIAB-NT-24	0.90$_{(24hrs)}$	2.35$_{(72hrs)}$	0.40$_{(72hrs)}$
NIAB-NT-25	0.70$_{(24hrs)}$	3.02$_{(96hrs)}$	1.00$_{(72hrs)}$
NIAB-NT-26	0.5$_{(96hrs)}$	2.14$_{(72hrs)}$	0.40$_{(96hrs)}$
NIAB-NT-27	1.10$_{(72hrs)}$	1.70$_{(96hrs)}$	0.40$_{(72hrs)}$
NIAB-NT-28	0.80$_{(24hrs)}$	0.93$_{(24hrs)}$	0.70$_{(48hrs)}$
NIAB-NT-29	---**	0.80$_{(24hrs)}$	0.40$_{(96hrs)}$
NIAB-NT-30	0.10$_{(48hrs)}$	1.05$_{(72hrs)}$	---**
NIAB-NT-31	0.90$_{(72hrs)}$	0.89$_{(24hrs)}$	0.30$_{(96hrs)}$
NIAB-NT-32	0.80$_{(72hrs)}$	1.05$_{(24hrs)}$	0.30$_{(96hrs)}$
NIAB-NT-33	2.9$_{(96hrs)}$	0.97$_{(72hrs)}$	0.60$_{(96hrs)}$
NIAB-NT-34	1.50$_{(24hrs)}$	0.92$_{(96hrs)}$	0.70$_{(72hrs)}$
NIAB-NT-35	2.7$_{(96hrs)}$	0.86$_{(24hrs)}$	0.40$_{(48hrs)}$
NIAB-NT-36	0.90$_{(24hrs)}$	1.32$_{(48hrs)}$	0.50$_{(72hrs)}$
NIAB-NT-37	---**	0.71$_{(72hrs)}$	0.80$_{(72hrs)}$
NIAB-NT-38	0.70$_{(48hrs)}$	0.90$_{(24hrs)}$	0.40$_{(48hrs)}$
NIAB-NT-39	0.40$_{(24hrs)}$	1.22$_{(72hrs)}$	0.40$_{(48hrs)}$
NIAB-NT-40	0.50$_{(24hrs)}$	1.43$_{(96hrs)}$	---
NIAB-NT-41	1.00$_{(24hrs)}$	1.23$_{(72hrs)}$	0.60$_{(48hrs)}$
NIAB-NT-42	3.80$_{(72hrs)}$	2.74$_{(48hrs)}$	1.00$_{(72hrs)}$
NIAB-NT-43	3.40$_{(72hrs)}$	3.00$_{(96hrs)}$	2.00$_{(72hrs)}$

** Negligible production of lysine

In molasses based medium M-II, NIAB-NT-8 produced 2.62g/L of lysine. Likewise, NIAB-NT-13 produced the 2.20g/L of lysine after 24 hours of incubation in the same medium. Moreover, production of 2.35g/L was recorded by NIAB-NT-24 after 72 hours incubation. Like M-I, the NIAB-NT-42 and NIAB-NT-43 produced maximum quantities of lysine *i.e*. 2.74 (48 hours incubation) and 3.00g/L (96 hours incubation), respectively. However, in molasses based medium M-III, NIAB-NT-43 and NIAB-NT-4 produced lysine in quantities of 1.50 and 2.20g/L, respectively, after 72 hours of incubation period.

Table II.- Time scale production of selected mutants in glucose trypticase medium (L-6 Medium).

Mutants	Lysine Production (g/L			
	24 hr	48 hr	72 hr	96 hr
NIAB-NT-2	1.03	0.00	3.56	1.30
NIAB-NT-7	0.68	1.08	1.30	2.32
NIAB-NT-8	0.96	2.51	2.65	2.18
NIAB-NT-10	0.38	1.99	2.71	2.09
NIAB-NT-12	0.85	1.87	1.54	1.06
NIAB-NT-13	0.60	1.79	0.00	4.35
NIAB-NT-14	1.00	1.15	1.83	2.36
NIAB-NT-15	1.00	3.98	5.00	2.66
NIAB-NT-16	1.02	2.38	1.23	0.99
NIAB-NT-17	1.02	1.93	2.90	1.67
NIAB-NT-18	0.26	2.17	2.82	1.54
NIAB-NT-19	0.43	2.05	2.60	2.11
NIAB-NT-20	0.69	1.00	1.09	0.80
NIAB-NT-26	0.63	0.39	2.16	1.80
NIAB-NT-32	0.89	1.46	2.19	2.00
NIAB-NT-41	0.91	1.26	4.60	2.85
NIAB-NT-42	0.68	1.20	3.16	2.98
NIAB-NT-43	1.80	1.80	3.19	3.38

On the potential of their lysine production in molasses media, 16 mutants were selected and tested in glucose medium, L-6. The medium contains glucose and can validate the results more effectively. There is concern when we use black steep liquor as it contains some toxic substances that can affect the growth kinetics of the microorganism. However, L-6 medium is used to eradicate those negative effects and potential of mutants to produce lysine can be assessed more effectively. Almost all of them produced lysine ~1.0g/l. However, the best production was observed up to a level of 5.0g/l (Table II). Apart from lysine, glutamic acid was also produced frequently and its quantities found in the fermentation broths ranged 4.1-10.1g/l (data not shown). Lysine production of 3.56, 5.00, 4.60, 3.16 and 3.19g/L was recorded by NIAB-NT-2, NIAB-NT-15, NIAB-NT-41, NIAB-NT-42 and NIAB-NT-

43, respectively.

The results showed varying trend as NIAB-NT-15 was not able to produce appreciable quantities of lysine in molasses based medium, however, it gave maximum production in glucose based medium. The mutants like NIAB-NT42 and NIAB-NT-43 nearly produced lysine in all media that show their vigor and potential to be used further for the improvement purpose.

In the nutshell, about two-fold increase was observed in lysine production by mutant as compared to its parent, NIAB SM-3. Similar results have been reported about a wild strain of E. coli by Costa-Ferreira and Duarte (1992) and Nadeem *et al*. (2002). The improvement of bacterial strains is essential for increasing the production of amino acids. Difference in production may be due to extent of mutagenesis or media formulations, and it might have been the main factor that mutants were unable to produce good quantities of lysine in M-III as compared to other media. A similar study using NTG and AEC was also done by Guha *et al*. (1984). Their bacterial strain produced 24g/L of L-Lysine after mutation. Likewise, Ikram *et al*. (2001) improved glutamic acid producing E. coli strain and observed two fold increase in lysine production. Later, Sattar *et al*. (2008) utilized fermented yeast sludge with *Brevibacterium flavum* and observed enhanced lysine concentration (Shah *et al*., 2002; Ahmed *et al*., 2010).

The future prospects of current study include the utilization of tools of genetic engineering for hyper-production of amino acids from mutant strains of bacteria. Moreover, the agro-industrial wastes can be utilized for media preparation. The different techniques can also be applied for enhancement of biosynthetic and transport capacity, which is beginning to have a great impact on the amino acid industry.

CONCLUSIONS

Amino acid production enhanced many folds during last two decades, mainly due to strain improvement which includes classical mutagenesis, genome mastering and media optimization. In present study a two-fold increase in lysine production was observed due to NTG mutagenesis for strain improvement. Mutants like NIAB-NT-15, NIAB-NT-41, NIAB-NT-42, NIAB-NT-2 and NIAB-NT-43 produced 5.00g/L, 4.60 g/L, 3.80 g/L, 3.60 g/L and 3.38 g/L, of lysine, respectively. The production of lysine could further be enhanced with optimization of fermentation conditions and growth kinetics under fed and batch culture methods.

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