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Enhanced L-Lysine Production by an *Escherichia coli* Mutant WARN 30522 after MNNG Treatment

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ABSTRACT

One of the *Escherichia coli* isolates, SW30 NIAB, from irrigation water channel, originally capable of producing 1.8 g/L of glutamic acid was gradually improved through mutation for lysine production. The culture was exposed to 100 µg/mL MNNG for 90 minutes. The mutant cells showed resistance against S-(β-aminoethyl)l-cysteine (AEC), a lysine analogue, and produced lysine (0.1-0.5 g/L). One of three mutants, AR305, was again exposed to MNNG. Three mutants, thus formed could produce 13-15 g/L of lysine.

Key Words: Amino acid; Fermentation; *Escherichia coli;* MNNG; AEC; Lysine

INTRODUCTION

Amino acid fermentation has reached a stage where it is playing a vital role for the supply of natural amino acids at industrial level. The discovery of glutamic acid producing bacterium, *Micrococcus glutamicus* (later renamed as *Corynebacterium glutamicum)* gave a new dimension to amino acid production. This break through laid the foundation for other researchers who lately reported many bacteria involved in amino acid fermentation (Kinoshita, 1999). The development of better microbial strains together with multidimensional approaches might be expected to improve the economics of fermentation process (Costa-Ferreira & Duarte, 1992). The artificial distortions of metabolism can result in the over-production of particular amino acids. Quick improvement in the microbial production strategies of amino acids has resulted from the interest in the nutritional applications of amino acids such as L-lysine. Apart from its role as a feed supplement, L-lysine along with some other amino acids like aspartic acid is used extensively in the pharmaceutical industry in the formulation of diets with balanced compositions and in amino acid infusion (Hirose & Okada, 1979; Costa-Ferreira & Duarte, 1992).

A great variety of microorganisms, including auxotrophic as well as regulatory mutants, has been reported to over-produce lysine (Tosaka & Takinami, 1986). The selection of such mutants has led to isolation of high producers, which are used for industrial production of lysine, glutamic acid, threonine and a variety of other amino acids (Han *et al.,* 1991). The lysine analogue S-(β-aminoethyl)-L-cysteine (AEC) can substitute for lysine in feedback inhibition. Thus, a mutant capable of exhibiting resistance against AEC may accumulate reasonable amount of lysine in the fermentation medium (Han *et al.,* 1991; Lu *et al.,* 1994).

In an earlier study, we had isolated a strain, *Escherichia coli* SW30 NIAB, from local irrigation water channel, which could produce glutamic acid (1.8 g/L). This isolate did not produce lysine but its mutant could produce lysine giving a yield of 0.1-0.5 g/L (Ahmad & Nadeem, 1993). The present research work was aimed at enhancing lysine production and describes the stepwise improvement of parent strain *E. coli* SW30 NIAB through mutation in this regard.

MATERIALS AND METHODS

The parent strain, *E. coli* SW30 NIAB, was inoculated in 50ml of minimal medium CGXII (Keilhauer *et al.,* 1993) adjusted pH 7.2 with KOH in 250mL Erlenmeyer flask and incubated overnight at 30˚C and 150 rpm. The population density of harvested cells was 190 NTU, with optical density 1.090 at 550 nm and 3 x 108 cells/mL and a pH of the medium 4.1. In order to observe the inhibitory effect of AEC against the wild type cells, cup plate bioassay technique was applied (Ahmad & Saba, 1993). The medium CGXII with 2% agar was used as basal plate- as well as seeding-medium. A 0.2 mL culture grown overnight in nutrient broth with population density of 2 x 108 cells/mL at a pH 8.2 was added to 10ml of the seeding layer. The bioassay cups (rings) were of 8 mm dia with 0.8 mL capacity. These were filled with different concentrations of AEC ranging from 625 µg/mL to 5,000 µg/mL prepared in 0.1 M phosphate buffer (pH 7.0). The incubation was carried out at 30˚C and inhibition zones were measured after 48 h (Yaqoob *et al.,* 1999).

Mutagenesis. The cells of parent strain, *E. coli* SW30 NIAB, grown in CGXII medium (Keilhauer *et al.,* 1993) with an approximate population density of 3 x 108 cells/mL were used for mutagenesis. The cells were pelleted at 3,000 rpm for 15 minutes, washed twice with 0.1M Citrate buffer

(pH 5.5) and then suspended in 4 mL of the same buffer. To this cell suspension was added 0.2 mL of 2 mg/mL MNNG (final concentration 100 µg/mL) and incubated at 37˚C at 150 rpm for 90 minutes. The cells were washed with the same volume of phosphate buffer and their dilutions were made. Then, 0.1 mL of each dilution was spread on Nutrient agar plates in triplicate and incubated overnight at 30˚C.

Selection of AEC-resistant mutants. The MNNG treated cells appearing on the plates were counted next day. Each colony was picked with sterile tooth pick and replica plated in triplicate. Three types of plates were used for replica-plating; type I contained simple nutrient agar medium, type II CGXII plus agar; whereas, the third type of plates contained in addition to it a solution of AEC with a concentration of 1.0 mg/mL dissolved in the same phosphate buffer. The plates were incubated for 72 h at 30˚C. The colonies appearing in type III (CGXII + Agar + AEC) were counted and stock cultured.

Selection of lysine-producing mutants. The above colonies, resistant to AEC, were inoculated in a glucose based production medium (Costa-Ferreia & Duarte, 1992). Growth was maintained in 50 ml per flask (Erlenmeyer, 250 mL) at 30˚C at 150 rpm up till 96 h, during which time the cell free fermentation broth was intermittently monitored for lysine production after every 24 h. The qualitative estimation of amino acids was done by paper chromatography (Nadeem *et al.,* 1997). Followed by paper electrophoresis. The rough estimation of lysine was done by eluting its coloured spots in methanol and reading the optical density at 550 nm and was compared with the standard. Afterwards, the fermentation broth of the strains reflecting promising results was assessed through amino acid analyzer.

RESULTS AND DISCUSSION

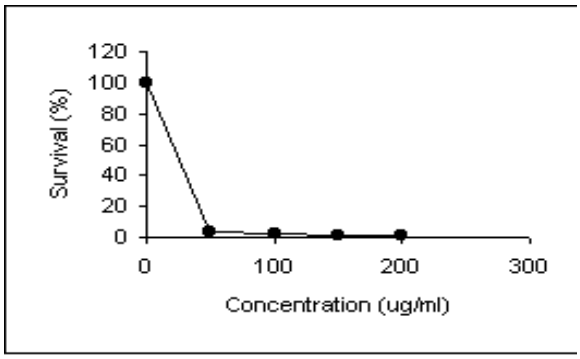
In the present study, the lysine productivity was gradually increased through repeated exposure to MNNG, which enabled the bacteria to display resistance against AEC. During the first phase of mutation studies (Yaqoob *et al.,* 1999), three mutants were isolated which were able to produce 0.1-0.5 g/L of lysine in contrast to a reference strain, *Corynebacterium glutamicum* ATCC 21798 (Table I). Among these isolates, AR-305 was selected for further improvement studies. The minimum inhibitory concentration (MIC) of AEC for the organism was 1250 µg/mL.

Fig. 1 shows survival curve of *E. coli* SW30 NIAB against different concentrations of MNNG in glucose medium. Nearly 2-3% was the survival rate of MNNG-treated cells after a 90-minute exposure to a concentration of 100 µg/mL. The cells that survived this dose were screened for their resistance against AEC. The 32 AEC-resistant mutants obtained were then cultivated in a fermentation medium. Three of the mutants produced nearly 13-15 g/L of lysine; two producers WARN 30000 and WARN 30522

Table I. Lysine production (g/L) in the wild type strain (*E. coli* SW30 NIAB), AEC-resistant mutants derived from the parent strain by MNNG treatment and ATCC reference strain

Strain	Growth period (Hrs.)		
	48	72	96
E. coli SW30 NIAB (wild type)	Nil	Nil	Nil
AR-302	Nil	0.3	0.3
AR-303	Nil	0.5	0.5
AR-305	Nil	0.1	0.5
Corynebacterium glutamicum ATCC 21798 (Reference)	2.0	4.8	4.8

Fig. 1. Survival curve of *E. coli* NIAB SW-30 against different doses of NTG after 90-minute exposure



gave maximum lysine production after 72 h of growth; whereas, the third one, WARN 30005, produced its best results after 48 h incubation (Table II).

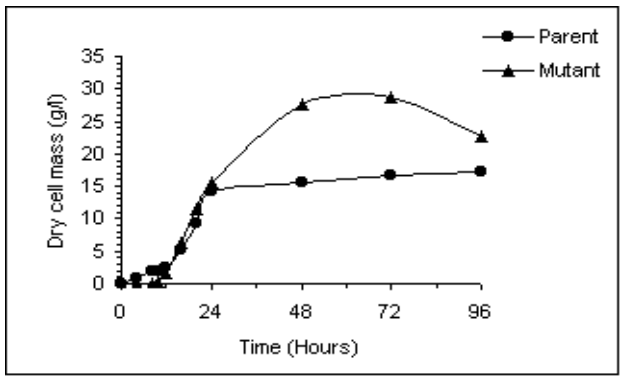
Table II. Amino acid production by AEC-resistant mutants derived from AR-305 as a result of repeated MNNG treatments

Mutants	Amino acids (g/L)			
	Lysine	Isoleucine	Tyrosine	Cysteine
WARN 30522	14.97	0.30	0.11	-
WARN 30005	13.88	0.34	0.12	0.10
WARN 30000	13.39	0.47	0.16	0.13

The most common method applied so far for overproducing amino acids is mutation and selection based on molecular biology. Analogue-resistant-mutant isolation is one of the methods successfully developed for better results. Obviously, resistance to AEC does not necessarily mean the overproduction of lysine as shown by our results. It is only a method successfully developed for the same purpose and it has yielded higher production of arginine, lysine, tryptophan, threnine etc. utilizing different bacterial strains (Aida, 1986).

Fig. 2 shows the growth curve of *E. coli* SW30 NIAB (parent) and that of its mutant, WARN 30522 exhibiting changes in dry cell mass (g/L) during growth over a total period of 96 h. Growth curves illustrate that lag phase of the

Fig. 2. Comparison of growth patterns of the parent, NIAB SW-30, and that of its mutant, WARN 30522, in complex media



parent was shorter, lasting about 4 h, than the mutant, which took more than twice as much time as the parent. In case of the parent, there was a sharp rise in growth during the first 24 h after which the rate of production was almost stable. The maximum growth (16.7 g/L dry cell weight) was, however, attained by the 72 h of incubation after which there was slight decrease in growth rate indicating that the death phase had started. In case of the mutant, the lag phase lasted for almost 10 h after which there was a quick rise in population upto 48 h. The maximum growth (28.6 g/L dry cell weight) was, however, observed by the 72 h.

Our research programme has been focused on developing a strain from local environment, which could produce lysine for poultry feed which is one of the leading industries in Pakistan. Since no amino acid is being produce locally, huge amount of foreign exchange is spent every year for import of amino acids, which are important ingredients of poultry feed. The present report is an attempt to develop a bacterial strain that could be used to produce lysine and hence meet economic needs of the country.

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