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INDUCED MUTATION AND SELECTION OF A HIGH YIELDING STRAIN OF MICROCOCCUS GLUTAMICUS FOR L-LYSINE PRODUCTION

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ABSTRACT

The objective of this investigation was to develop a new auxotropic mutant from a regulatory mutant and to examine its potency for L-lysine production. A high L-lysine yielding strain *Micrococcus glutamicus* AB_{200} was developed from a regulatory mutant *Micrococcus glutamicus* AB_5 by subsequent mutagenic treatment with UV rays and methyl methane sulfonate respectively.
Treatment of UV rays on the regulatory mutant AB_5 resulted in a mutant strain AB_{50} gives the maximum yield of L-lysine (5.5mg/mL) than the regulatory mutant (0.9 mg/mL). The mutant strain AB_{50} on further treatment with a chemical mutagen methyl methane sulfonate resulted to another mutant *Micrococcus glutamicus* AB_{200} which produced the highest yield of L-lysine (7.2mg/mL) *Micrococcus glutamicus* AB_{200} was an auxotropic mutant which requires biotin for growth as well as production of L-lysine. The L-lysine producing capacity of auxotropic mutant was tested after maintaining the strain in three different media of which the mutant was stable in a media having the composition of 5% glucose, 0.6% urea, 0.2% K_2HPO_4. 0.025% $MgSO_4$. $7H_2O$, 2μg/mL biotin.

KEYWORDS

Micrococcus glutamicus AB_{200}, *Micrococcus glutamicus* AB_{50},Yeast, Biotin, L-lysine, Auxotroph, UV rays, MMS.

INTRODUCTION

The gram positive bacteria *Micrococcus glutamicus* is widely used for the industrial production of different amino acids.1 The fermentation production of amino acids started in the fifties(1956-57) with the discovery of L-Glutamic acid producing bacteria by Japanese scientists. (2, 3). Since then, most of the development work was devoted to the improvement of mutant strains involving classical and modern microbial genetics (4, 5), thereby increasing the efficiency of commercial L-lysine production. Microorganisms employed in microbial production of amino acids are divided into four classes: wild type, auxotropic mutant, regulatory mutant and auxotropic regulatory mutant strain. (4,7,8). Major improvement in the productivity of fermentation processes are generally ascribed to be the development of superior strains via mutation.(9) Selected mutant strains with improved productivity characteristics are used today in industry for L-lysine production, which are obtained by various mutagenic treatments.

Lysine is one of the major products, with worldwide production of about 4, 00,000 tons per year and annual market increase of about 10-15%.(10). It is mainly used as feed additives in the animal feed industry. As a fine ingredient, it is utilized in human medicine, in cosmetics and in the pharmaceutical industry particularly as ingredients of infusion solution for pharmaceutical applications. (8).

In order to meet the demand of L-lysine, four procedures have been proposed. i) Extractive isolation, ii) Chemical Synthesis, iii) enzymatic catalysis, iv) fermentative production. The microbial production of amino acids has got one advantage over the others is the fact that amino acids produced by the former process are of natural i.e. of L-form. Thus the stereochemistry of amino acids makes the microbial production advantageous compared with the synthetic process. (11). Considering the economic importance of L-lysine and superiority of the microbial production of this amino acid, the major aim of this study is to develop a new auxotropic high L-lysine yielding mutant strain of Micrococcus glutamicus from a regulatory mutant which can be used for large scale production of L-lysine by fermentation process using indigenous raw materials available in our country especially from the stand point of saving production cost so that the industrial demands of L-lysine could be met. (12). So in our present study , UV radiation as physical mutagen followed by treatment of methyl methane sulfonate(MMS) as chemical mutagen have been chosen for the development of high L-lysine yielding auxotropic mutant by induced mutation.

MATERIALS AND METHODS

Microorganism: Micrococcus glutamicus ATCC13032 is a gram positive non-sporulating and non-motile bacterium, with polymorphic short rods producing yellowish colonies (13) was used for this work. The organism used was Micrococcus glutamicus AB_1 which was obtained by natural screening method, found to produce a small amount of L-lysine. A regulatory mutant and an auxotropic mutant derived from it were used in this study. To maintain the strain nutrient agar medium was used and the strains were sub cultured once in every three weak and after 48h of incubation the cultured was stored at 4° C.

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Media composition: i) Complete Medium: glucose: 1%, peptone: 0.5%, beef extract: 0.3%, yeast extract: 0.1%, pH: 7.2(before sterilization). ii) Medium for L-lysine production: glucose: 5%, Urea: 0.8%, K_2HPO_4:0.1%, $MgSO_4.7H_2O$:0.025%, yeast extract: 0.2%, pH: 7.0. iii) Minimal medium(MM): glucose: 2%, urea: 0.2%, K_2HPO_4:0.1%, $MgSO_4.7H_2O$:0.025%, Biotin 0.1µg/mL, pH: 7.2. Solution of glucose was sterilized before mixing biotin. This medium was used through the mutation study.

ANALYSIS OF AMINO ACIDS

Descending paper chromatography was employed for detecting L-Lysine in culture medium and was run for 16 to 18 h on Watmann No: 1 chromatographic paper. Solvent system: n-butanol:acetic acid: water 2:1:1. The spots were visualized by spraying with a solution of 0.2% ninhydrine in acetone and quantative estimation of L-lysine in the suspension was done using colorimetric estimation method.

RECOVERY AND IDENTIFICATION OF L-LYSINE

For the identification of L-lysine the fermentation broth after separation of cells was adjusted to pH = 5 with HCl and filtered. The filtrate was then treated with active charcoal to remove the colored impurity. After filtration the clear filtrate was finally passed through a Dowex 50(H^+ type) column in which the amino acid was absorbed. After washing with water and elution with 0.24(N) HCl, the elute was further passed through an amberlite (H^+ type) for elimination of chloride ion. The elute was concentrated by evaporation in vacuum and the product was obtained in a crystalline form in the cold. The pure material was proved to be L-Lysine both by chemical test, optical rotation and by paper chromatographic method.

Insoluble picrate derivative of L-Lysine was prepared and melting point of the derivative 266°C. [4].

Elemental analysis of the pure material gave the following values: C – 49.31%, H – 9.63%, N – 19.17%, O – 21.88%. Whereas the molecular calculation shows the following values: C – 49.29%, H – 9.65%, N – 19.16%, O – 21.89%. Optical rotation was estimated as $(L)^{25}_{D}$ =20.1 (C=2, 5N HCl)

These data agreed with those of L-lysine.

TREATMENT WITH UV RAYS

Classical mutagenesis of microorganism were exposed to ultraviolet light irradiation. The source of radiation was a Hanovia germicidal lamp (15 watt). 2mL of bacterial suspension containing 10^6 cells/ mL were taken in a Petridis (5 cm diameter) and exposed to UV rays from a distance of 12cm for different period of time (12) (Table1). The treated M. glutamicus cells were and plated on agar medium to develop discrete colonies, which were incubated at 29°C for 48 h and selected colonies were transferred to agar slant containing yeast extract for proper growth (48 h,29°C) and stored at 4°C.

TREATMENT WITH METHYL METHANE SULPHONATE (MMS)

Cell suspension (containing 3.5×10^7 cells/mL)was added to 9 mL methyl methane sulfonate (MMS) solution of different concentration was prepared from a stock solution having conc. 0.2M resulting in dilution of MMS to 0.1M and0.05M. After a certain period of incubation (Table2), in each case 1mL of sample was diluted in water. The diluted bacterial cells were plated on CD agar medium and kept at 29°C for 48h for the development of discrete colonies. The colonies were transferred to agar slant, incubated at 29°C, 48 h for proper growth and finally stored at 4°C.

The cells selected from different stages of treatment with mutagens (UV rays and MMS) were finally tested for L-lysine production for surface culture fermentation process.

RESULTS AND DISCUSSIONS

Effect of UV rays

The bacterial suspension of Micrococcus glutamicus AB_{15} was treated with UV rays (15watt) for different period of time (table:1). The scoring of survivor and productive variants has been depicted in fig:1. Complete inactivation of cells took place in 90 minutes. Number of productive variants increase up to 45 minutes of treatment and then number of productive variants decreases with increasing period of exposure.

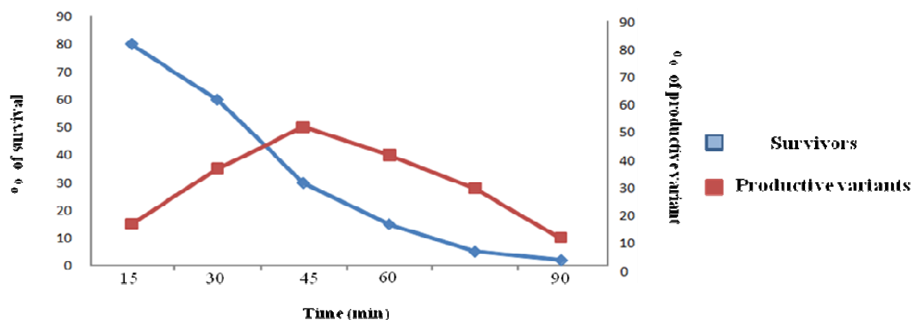


Figure 1: The scoring of survivors and productive variants

A maximum of 15 productive were obtained on exposure to UV rays for 45 minutes. After different period of UV rays 49 isolates were selected for L-lysine production. (Table1). It was observed that the mutant Micrococcus glutamicus AB_{50} exhibit the highest L-lysine producing capacity (6.5mg/mL). This strain was further subjected to chemical mutation using MMS as chemical mutagen.

Table 1

Relation between nutritional requirements of auxotroph obtained by exposure of M. glutamicus AB_5 to UV rays and their amino acid accumulation

Period of exposure to UV rays	Total No of auxotrophs	Extracellular amino acid pattern					Requirements			□*
		L-lysine			Other amino acids		Yeast extract	Amino acid	Vitamins	
		Similar to parents	More than parents	Less than parents	Glutamic acid	Methionine				
15	6	-	2	1	2	1	2	3	6	1
30	9	2	3	-	2	2	1	4	9	2
45	15	3	6	2	3	1	2	4	15	2
60	11	2	5	-	2	1	3	5	11	1
75	6	1	2	1	2	-	2	2	6	2
90	2	-	1	-	1	-	3	1	2	1

**frequency of spontaneous reversion X 10^3*

Effect of methyl methane sulfonate

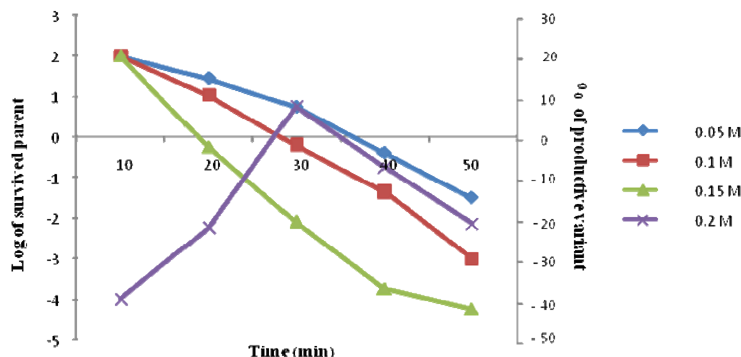


Fig 2: Survival of *M. glutamicus* AB_{50} . obtained by treatment with different concentration of MMS and development of productive variants at different intervals of time.

The bacterial suspension of Micrococcus glutamicus AB_{50} was then treated with MMS of varying concentration. A comparative study was made on the survival of bacterial cells of *Micrococcus glutamicus* (Fig:2) shows that MMS at a concentration of 0.2M, 0.1M, 0.05M. The maximum development of productive variants occurred when the period of treatment with the mutagen at a conc. of 0.1M at15 minutes giving the survival of 0.04%.

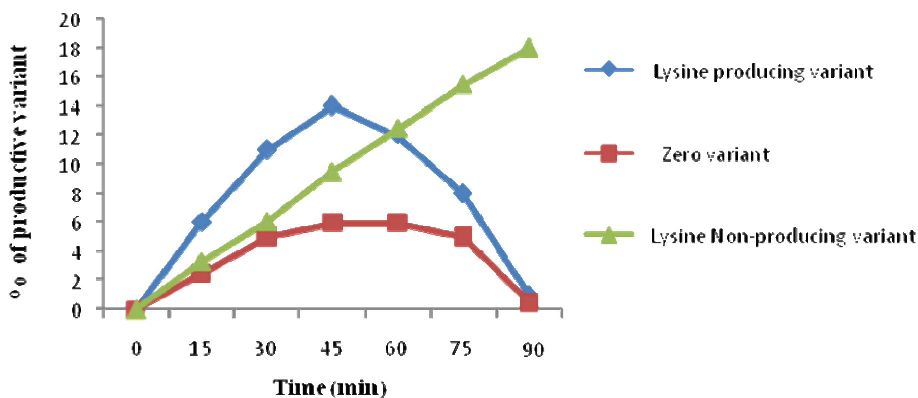


Figure 3: Distribution of productive variant by MMS treatment

The distribution of the productive variants of the treatment has been given in Fig: 3. the development of auxotropic L-lysine producing variants showed a maximum followed by the declination, while non L-lysine producing variants showed an increasing pattern even up to 45 minutes with 0.1M MMS solution. Thus we have isolated 120 strains after the treatment of MMS at different dilution which L-lysine producing variants, L-lysine non-producing variants and zero variants, among which fifty auxotropic mutants isolated, were tested for L-lysine production. Micrococcus glutamicus AB_{200} exhibits highest L-lysine producing capacity in fermentation medium (7.5 mg/mL). The production of parent strain AB_{15} (a regulatory mutant) was 0.9 mg/mL.

Table 2
Relation between nutritional requirements of auxotroph obtained by treatment of M. glutamicus AB_{50} with Methyl Methane Sulfonate (MMS) and their amino acid accumulation

MMS (M)	Period of exposure to MMS	Total No of auxotrophs	Extracellular amino acid pattern					Requirements			*
			L-lysine			Other amino acids		Yeast extract	Amino acid	Vitamins	
			Similar to parents	More than parents	Less than parents	Glutamic acid	Methionine				
0.20	15	9	3	1	2	2	1	1	2	9	1
0.10	30	18	5	3	3	5	2	3	3	18	2
0.05	45	23	3	4	4	7	5	2	3	23	2

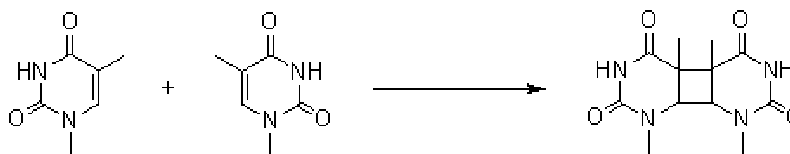
****frequency of spontaneous reversion X 10^3***

Table:2 shows the types of auxotroph and the amino acid accumulation by the mutants obtained in the medium under condition of MMS treatment. The auxotropic mutants exhibited varied pattern of extra cellular amino acids. A number of them (22%) maintained the parent pattern of excretion and about (18%) excreted Less L-lysine. Two different amino acids, viz. glutamic acid and methionine were found to be excreted by the other auxotroph screened. Out of 50auxotroph, 28 excreted 1-5mg/mL L-lysine and all of them required vitamins for growth and amino acid accumulation. 14auxotroph produces L-glutamic acid and 8 produces methionine. They also required vitamins for their growth and amino acid accumulation. Auxotrophs are classified as required yeast extract, amino acids and vitamins.

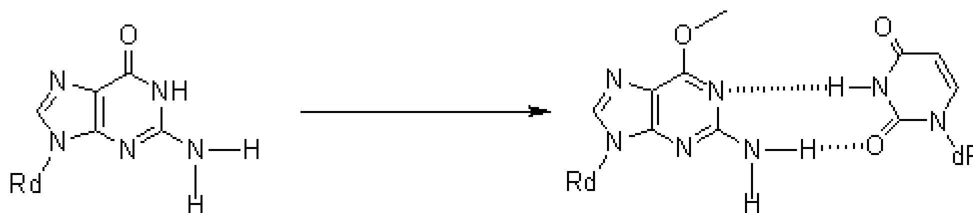
The mutational study showed that mutagenic effect of UV rays and MMS on Micrococcus glutamicus have been well distinguished from the nature of survival curve. Both the mutagen shows a sharp killing effect.

A total of 99productive strains were obtained and studied in the present work. It was observed that UV irradiation resulted in the fermentation of 56% productive variants and methyl methane sulfonate produces 63.2% productive variants.

UV rays caused mutation because the purine and pyrimidine bases in DNA absorb light strongly in the UV range (254 - 260nm) One of the effects of UV radiation on DNA is the formation of abnormal chemical bonds between adjacent pyrimidine molecules in the same strand or between pyrimidine of the opposite strands, of the double helix.



Scheme :1
Production of thiamine dimer by UV light irradiation. The two components of the dimer are covalently linked in such a way that the DNA double helix is distorted at that position.



Scheme :2
Methyl methane sulfonate (MMS) ,an alkylating agent, alkylates guanine.
dR= deoxyribose

On the other hand MMS is one of the diverse groups of alkylating agent that introduce alkyl groups (eg. $–CH_3$, $-CH_2CH_3$) on to the base at a number of locations. (scheme:2).
Most mutation caused by alkylating agents results from the addition of an alkyl group to the 6-Oxygn of guanine to produce O^6-alkylguanine. For example after treatment with MMS, some guanine are methylated to produce O^6-methylguanine. The methylated guanine pairs with thyamine rather than cytosine giving GC to AT transition. (scheme:2).
Both the mutagens altered the base so that a new base pair appeared in daughter cells in a later generation which might adversely or favorably affect L-Lysine production.[14].
In the present study, the superiority of UV rays irradiation is observed for the development of high L-Lysine yielding auxotroph of Micrococcus glutamicus whereas treatment with MMS favours the development of L-glutamic acid and methionine producing auxotroph.
In our study, the regulatory mutant AB_5 produces only 0.9mg/mL of L-lysine. Mutagenic treatment of this strain with UV produces mutant strain AB_{50} which produces 3.5mg/mL of L-lysine. After treatment with MMS another Mutant strain AB_{150} produces 7.2mg/mL L-Lysine. But further development of biotin requiring auxotroph, high L-Lysine producing mutant strain Micrococcus glutamicus AB_{200}, which can produce L-Lysine upto 8.1 mg/mL in the present condition of fermentation. So a mutagenic treatment developed a high L-Lysine yielding mutant strain of Micrococcus glutamicum from a low L-Lysine producing regulatory mutant. The newly developed strain can be used for large scale industrial production of L-Lysine using indigenous raw materials available in India. More over here is a constant attempt to optimize physical condition so that more lysine can be accumulated.

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