CHAPTER 2

INDUCED MUTATION AND SELECTION OF A HIGH YIELDING STRAIN OF *ASPERGILLUS NIGER* FOR BIOLEACHING OF SILICA AND IRON OXIDE FROM BAUXITE ORE AND SELECTION OF THE MEDIUM FOR THE MAINTENANCE OF MUTANT *ASPERGILLUS NIGER* X_1.

Different heterotrophic micro-organisms are widely used under laboratory conditions for releasing metals from various mineral raw materials. In contrast to the autotrophic bacteria, the heterotrophic bacteria and fungi require organic carbon for growth and do not derive any benefit from the degradation of the minerals which occurs because of their presence. This makes the heterotrophs less amenable for commercial operations than the autotrophs. However, some leaching processes connected with the activity of different heterotrophic micro-organisms seem promising for a real time application.

Among the micro-organisms that are effective in mineral degradation, more precisely, the biodegradation of bauxite, the role of **A.niger** is important. Although many of the wild strains of **A.niger** are having such effect, their efficacies are not considered to be optimum. Hence, research in the direction of improvement of the leaching ability of **A.niger** has continued. For the improvement of the leaching ability by any micro-organism following two pathways are adopted

(i) improvement of the parent strain, and

(ii) improvement of the environmental conditions.

So, the concerned organism should be studied to find out the better leaching capacity. This is the importance of the industrial microbiology. From the mycological stand point it can be said that search for new strains in nature or to search for strains which may arise as natural variants among the single spore colonies of the parent strain. But spontaneous mutation is

a very slow process and so this process of selection can never be the sole applicable technique in the field of microbial industry.

The alternative method is evidently to search for mutants which may be induced amongst the progeny of the parent strain, after treatment with mutagens. The idea of treating micro-organisms with various mutagens and to search for improved mutants among the surviving progeny has now been recognized as the best means to secure strains of improved potency. Kresling and Stern **(146)** first reported mutants of **Aspergillus niger** producing considerably more citric acid than the parent strain. They used UV-light and radium as mutagens. But the feasibility of these observations were not realised till 1945, when Demerec **(147)** reported a new mutant X-162 of **Penicillium notatum** showing improved yield of penicillin in comparison with the parent strain. Since this observation the process of induced mutation and strain selection has been used in improving the yield of various metabolic products.

In this present chapter our findings regarding the development of a mutant strain of **Aspergillus niger** for the leaching of silica and iron from bauxite ores are discussed. V.I. Groudeva and S.N. Groudev used mutant strain of silicate bacteria for leaching of silicon from the bauxite ores **(148-149).**

2. EXPERIMENTAL AND RESULTS :

Various samples of bauxite ores from Araku valley site of Orissa and M.P. were collected and crushed in a ball mill to 200 mesh size for silica and iron solubilization experiments.

A pure culture of **Aspergillus niger** isolated from sandy loamy soils were used in this experiment. This **Aspergillus niger** is capable of releasing silicon and iron from bauxite ores (9.8% and 10.1%). Where the silicon and iron are present as the major impurity (15%) in its free form as silica SiO_2 and Fe_2O_3.

2.1 Mutagenic Treatment :

The present culture used in the experiment was **Aspergillus niger** strain as selected out of sixty isolates of the fungus from various sources. Spore suspension was prepared in sterile normal saline water from the spores scraped from 10 days old Czapek Dox (CD) agar slants. The preparation was thoroughly shaken and filtered twice through sterile cotton to remove spore clumps. The concentration of the spore suspension was finally made up to 12 x 10^6/ml. The mutant strains were maintained on malt extract and yeast extract agar slants at 4°C. The Czapek-Dox agar medium was used for the mutational studies. The medium (FM) used for the fermentation of bauxite ores for leaching of silica and iron, consisted of 0.2% $NaNO_3$, 0.1%

KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4,7H_2O$ and 0.01% yeast extract. pH was adjust-ed to 4. It was sterilized at 121°C for 15 min. 4% glucose was sterilised separately and added to the medium aseptically.

When the cultures were needed for mutational studies, they were transferred to slants of malt-extract and yeast-extract agar and incubated at 30°C for 6 days for sufficient sporulation. Spore crops were harvested by washing the slants with sterile normal saline water and filtering the resulting spore suspension through several layers of sterile absorbent cotton. The spore density was adjusted to 12 x 10^6/ml. The spore suspension was used both for mutational studies and for the inoculation of the fermentation medium. Submerged culture fermentation was carried out using 200 ml polypropylene flasks (rotary shaker 100 rev./min.) containing 50 ml of the medium (F.M.) at 30°C for 6 days.

2.1.1 Treatment with Ethylene Imine :

Spore suspension (1 ml) was added to 9 ml of ethyleneimine solution of different concentrations resulting in the dilution of ethylene imine to 1:3000, 1:5000 and 1:7000. At an interval of 1 hour, 1 ml of the sample was diluted to 10 ml with sterile normal saline. The diluted spores were then plated in CD agar medium and kept at 30°C for 6 days to develop distinct colonies. After sporulation, colonies were transferred to malt extract and yeast extract agar slants and stored at 4°C.

2.1.2 Treatment with X-rays :

Two ml of the spore suspension (containing 12 x 10^6 spore/ml) taken in a petridish (5 cm diameter) and were exposed to X-rays (35 KV and 10 mA) at a distance of 10 cm for different periods of time. The treated **A.niger** spores were plated on the CD agar medium and plates were incubated at 30°C for 6 days. Selected colonies were transferred to malt extract and yeast extract slants and stored at 4°C.

The spores selected from different stages of treatment with mutagens (ethylene imine and X-rays) were finally tested for leaching of silica and iron from bauxite ores by submerged culture fermentation process.

2.2 Determination of silica and iron concentration :

At the end of fermentation, 50 ml of the fermentation broth was taken out of the flasks aseptically and centrifuged at 10,000 r.p.m. for 10 min in Sorvall RC-5 superspeed centrifuge at room temperature to remove biomass and unsolubilised ore matters. The clear solution was digested with 1:1 H_2SO_4 and after digestion the solution was diluted with demineralized water and filtered for separating SiO_2. The filtrate was made upto the mark in a 250 ml volumetric flask. The quantity of SiO_2 was determined by firing the residue in a muffle furnace at a temperature of 900°C for 1 hour and then by its subsequent hydrofluorisation with HF and H_2SO_4. The amount of Fe_2O_3 was estimated by titrating a definite volume of the above filtrate with standard $Hg_2(NO_3)_2$ solution. The amount of

Al_2O_3 content in the sample was determined by titrating the filtrate with standard EDTA solution using xylenol orange indicator **(150).**

2.3.1 Effect of Ethylene Imine Concentration :

A comparative study has been made on the effect of ethylene imine on the survival of spores of **A.niger**. It appears that(Fig.2.1) ethylene imine solution at a concentration of 1: 3000 shows greater killing effect with a steep slope in the survival curves while a concentration of 1:5000 gives moderate effect. The maximum development of productive variants occurs when the period of treatment with the mutagen is at a dilution of 1:5000 for 5 hours giving the survival percentage of 0.3% (Fig.2.1).The distribution of the productive variants of this treatment is shown in (Fig. 2.2).The development of (+) variants shows a maximum and then declines, while negative variants show an increasing pattern even upto 7 hours. In all, 1200 isolates after treatment with ethylene imine were tested for leaching of silica and iron from bauxite ores. **A.niger** AB 101 gave higher leaching capacity of silica and iron (22.7% and 25.2% respectively) in medium (FM). The parent strain **A.niger** AB leached only 9.8% silica and 10.1% iron from bauxite ores.

2.3.2 Effect of X-ray Treatment :

The spore suspension of **A.niger** AB 101 was treated with X-rays (35 KV and 10 mA) for different periods of time. The scoring of survivors and productive variants is depicted in

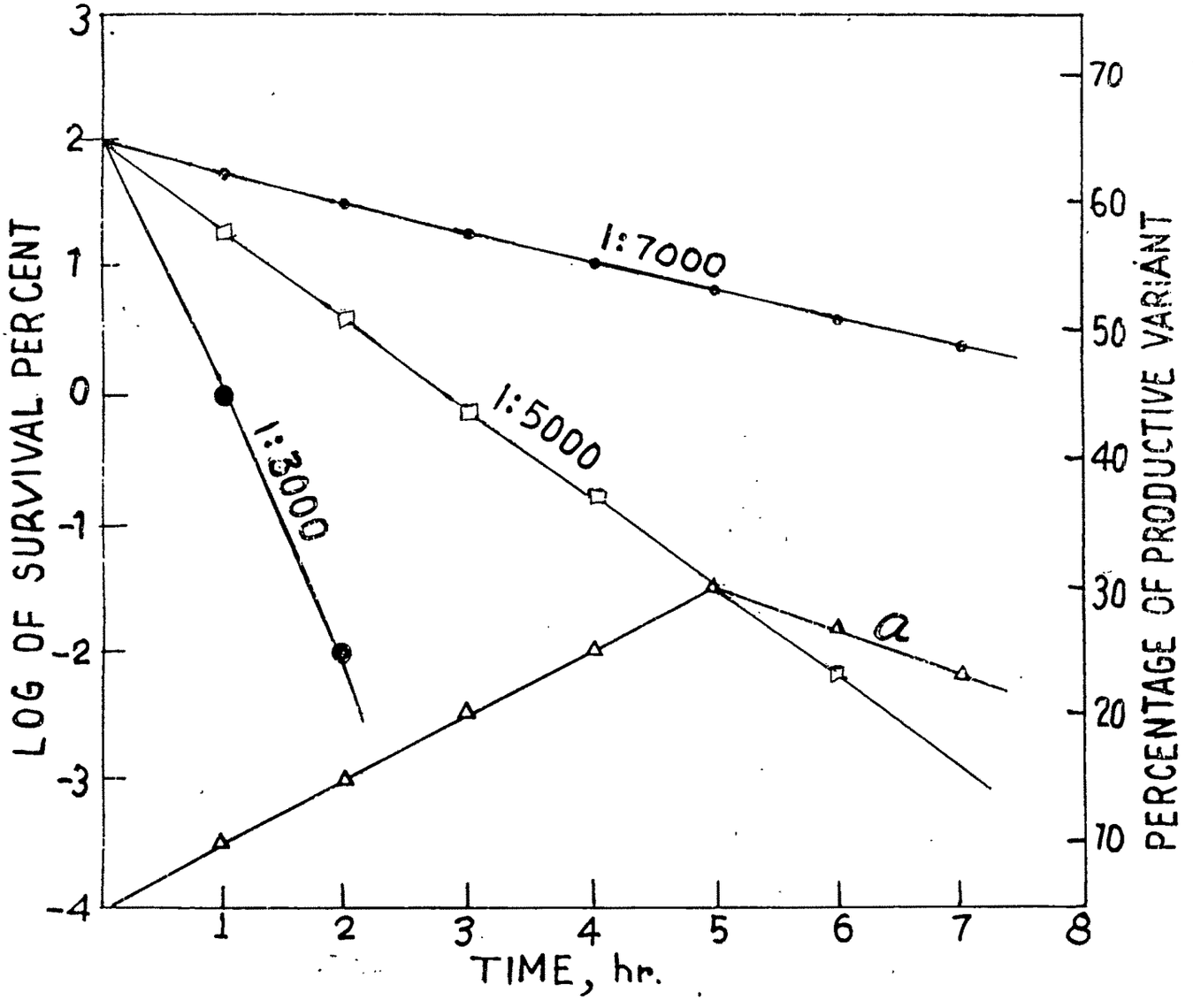

Fig.2.1 SURVIVAL CURVE OF A. niger AB OBTAINED BY TREATMENT WITH DIFFERENT DILUTIONS OF ETHYLENE IMINE AND THE DEVELOPMENT OF PRODUCTIVE VARIANTS AT DIFFERENT INTERVALS OF TIME. (CURVE a REPRESENTS DEVELOPMENT OF PRODUCTIVE VARIANTS FOR THE TREATMENT WITH 1:5000 DILUTIONS).

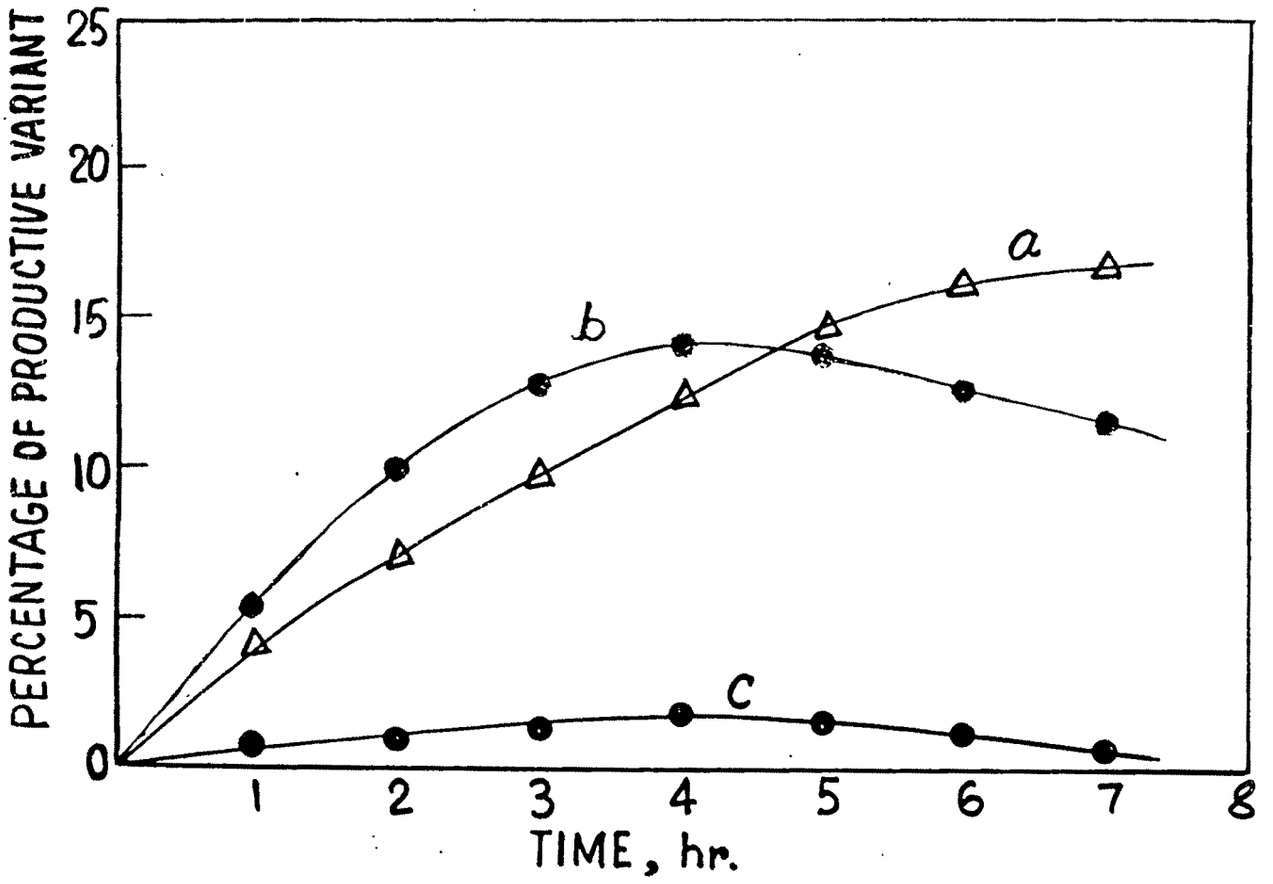

Fig.2.2 DISTRIBUTION OF PRODUCTIVE VARIANTS OBTAINED BY ETHYLENE IMINE TREATMENT [a, (-) VARIANTS, b, (+) VARIANTS AND c, (0) VARIANTS]

(Fig.2.3).Complete inactivation of spores took place in 120 min. Productive variants were detectable only upon treatment for 20 min. and their number increased with increasing period of exposure. A maximum of 30% productive variants were obtained on exposure to X-rays for 6 min. After different treatment with X-rays, 850 isolates were selected for leaching of silica and iron from bauxite ores, it was observed that the mutant **A.niger** X_1 gave 49.8% and 56.4%, respectively from bauxite ore in medium (FM). This strain was studied to standardise the cultural condition for leaching of silica and iron from bauxite ores.

The present study shows the mutagenic effects of ethylene imine and X-rays on spores of **A.niger** are well distinguished from the nature of the survival curve. Ethylene imine gives a survival curve of sharp killing ratio (exponential curve). There is also a sharp killing effect with X-rays.

A total of 501 productive mutants were obtained and studied in the present work. It was observed that ethylene imine treatment produced 35% productive variants including 15% (+) variants. X-ray irradiation results in the fermentation of 30% productive variants which comprised 10% more productive (+) variants.

The effect of ethylene imine, an alkylating agent is to modify the DNA nucleotide permanently by chemical modification and this alkylating process is accompanied by an enzymatic breakage of the alkylated DNA in vitro **(151-152)**. Therefore in course of self-duplication of DNA, this type of

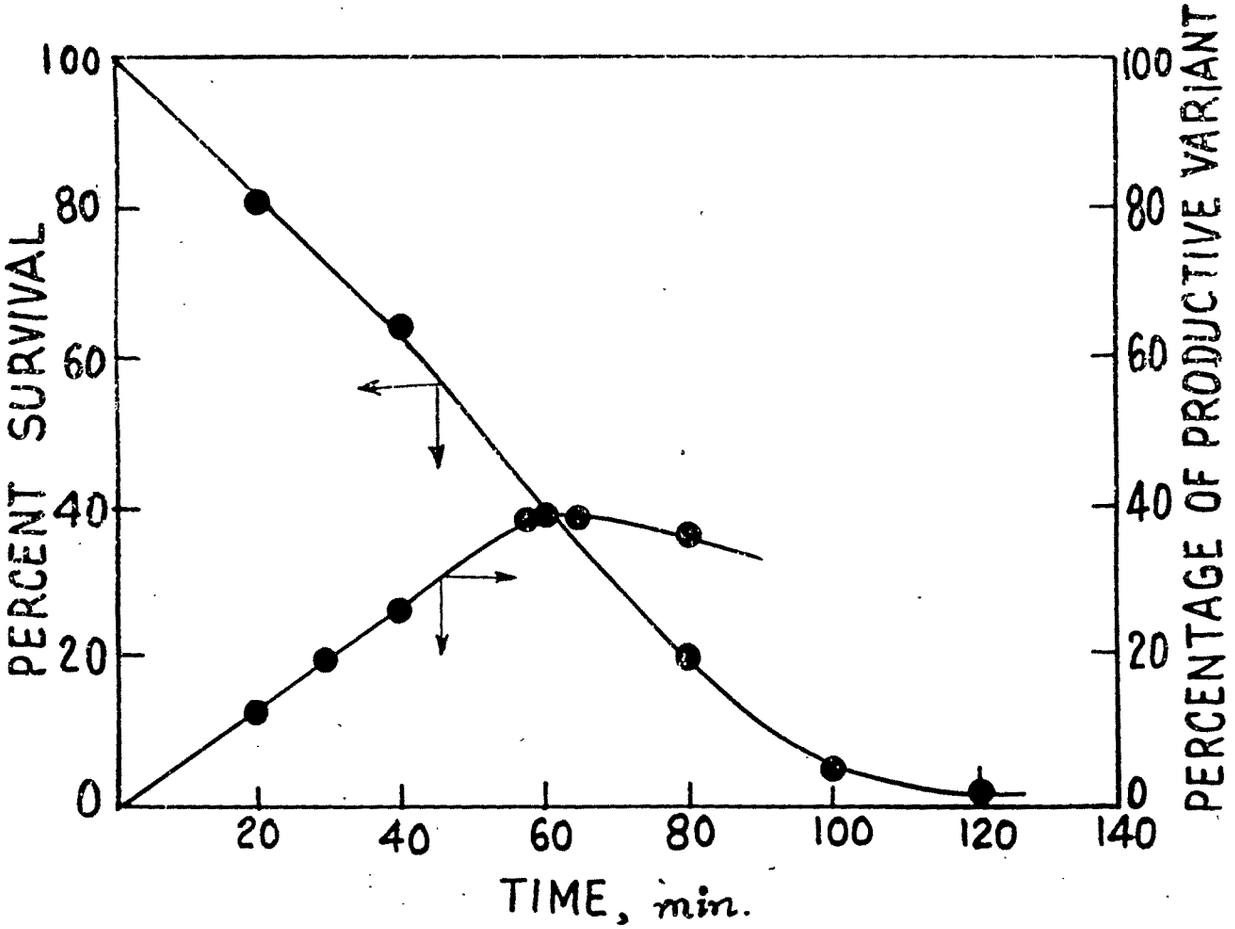

Fig.2.3 THE SCORING OF SURVIVORS AND PRODUCTIVE VARIANTS OBTAINED BY TREATMENT WITH X-RAY.

change will induce some permanent change in the DNA structure and as a result the stability of the mutant is very appreciable. These are supported by the stability test of the variants. The superiority of ethylene imine over X-rays as a mutagen for the development of a stable mutant, as documented elsewhere **(153)** is also observed in the present study.

2.4 Selection of the medium for the maintenance of the mutant A.niger X_1.

When the mutant **A.niger** X_1 obtained from X-rays irradiation was maintained in the original CD agar slant and subcultured in the same medium for 3 months, a degeneration of the mutant and decrease in the leaching of iron and silica from bauxite ore was observed. So, it was considered necessary to select a suitable medium for maintenance of **A.niger** X_1. The maintenance medium was selected from the following three media.

Medium I

Malt extract- 0.5% Yeast extract - 0.5%

Agar - 3% pH : 4.0

Medium II

Glucose - 5% KH_2PO_4 - 0.1%

KCl - 0.05% $NaNO_3$ - 0.2%

$MgSO_4.7H_2O$ - 0.05% $FeSO_4.7H_2O$ - .0001%

Agar - 3% pH - 4

Medium III

Glucose - 1% Peptone - 0.5%

Yeast extract - 0.5% Beef extract - 0.3%

pH - 4.0

Fermentation was carried out in the FM medium under the condition as described elsewhere (Chapter 2 Page No. 46 of the thesis). The results are shown in Table 2.1.

Table 2.I shows that the mutant **A.niger** X_1 subcultured in medium I for 10 months is relatively stable with respect to silica and iron leaching. So, medium I was finally selected for the maintenance of the mutant.

Table 2.I : Leaching of Silica and Iron by the mutant A.niger X_1 maintained in different media.

Maintenance medium	Silica and Iron leaching (%)																	
	Initial		After															
			1st month		2nd month		3rd month		4th month		5th month		6th month		8 month		10th month	
	Silica	Iron oxide	Silica	Iron oxide	Silica	Iron Oxide	Silica	Iron Oxide	Silica	Iron Oxide	Silica	Iron Oxide	Silica	Iron Oxide	Silica	Iron Oxide	Silica	Iron Oxide
I	49.8	56.4	49.8	56.4	49.7	56.3	49.9	56.5	49.7	56.4	48	54.1	47.5	54	46	51.1	45	50
II	49.7	56.5	49.8	56.4	47	53	43	49.1	40	48.2	35.7	42.3	30.5	40	27	34.2	22.1	28.2
III	49.9	56.4	48.1	54.2	45	52.1	40	50.9	36	46.7	32	40.1	29	39.1	22	30.1	18.2	26.8