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Strain improvement studies on L-aspaginase producing bacteria

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ABSTRACT
In the present study, we report the strain improvement studies on L-asparaginase producing a newly isolated Bacillus cereus MS-6. The UV induced mutant MUV-9 showed highest L-asparaginase activity (24.98 IU/ml). It was 7.84 fold of the parent strain. Further mutagenesis of this strain by NTG treatment could yield few mutants. Examination of L-asparaginase activity of the selected mutants showed that the mutant MNTG-7 showed further improvement in L-asparaginase activity (38.06 IU/ml) (Fig. 4.4). It was12.04 fold more of the wild strain (MS-6, 3.16). The EMS mutants (from parent MNTG-7) have not much difference between parent strain and EMS mutants.
Keywords: L-Asparginase, bacillus cereus, MS-6 and mutants

INTRODUCTION
A major potential therapeutic application of enzymes is in the treatment of cancer. Asparagianse has proved to be particularly promising in the treatment of acute lympho-cytic leukaemia. Tumour cells are deficient in aspartate-ammonia ligase activity, which restricts their ability to synthesize the normally non-essential amino acid, L-asparagine. Therefore they are forced to extract it from body fluids. The action of Asparaginase does not affect the functioning of normal cells, which are able to synthesize enough for their own requirements, but reduce the free exogenous concentration, and so induce a state of fatal starvation in the susceptible tumour cells (Sabu, A, 2003). A 60% incidence of complete remission has been reported in a study of almost 6,000 cases of acute lymphocytic leukaemia. This enzyme is administered intravenously.

L-asparagine+H_2O L-aspartate+NH_3

Strain improvement is an essential part of process development for fermentation products. Developed strains can reduce the costs with increased productivity and can possess some specialized desirable characteristics. Such improved strains can be achieved by inducing genetic variation in the natural strain and subsequent screening. Thus a major effort of industrial research in producing enzymes is directed towards the screening programs. Mutation is the primary source of all genetic variation and has been used extensively in industrial improvement of enzyme production (Ghisalba et al., 1984; Sidney and Nathan, 1975). The use of mutation and selection to improve the productivity of cultures has been strongly established for over fifty years and is still recognized as a valuable tool for strain improvement of many enzyme-producing organisms Paradee, A B(1973).

The industrial geneticist is rarely able to predict exactly what type of mutation is required to improve

the given strain. Hence a series of mutagenic treatments are carried out to develop a better yielding strain by trial and error. Various mutagenic agents such as ultraviolet rays (UV), N methyl-N'-nitro-N-nitrosoguanidine (NTG), X-rays, gamma rays, nitrous acid, ethyl methyl sulfonate (EMS) etc., are generally used for yield improvement studies. The ultra violet irradiation , Meyroth, J (1971) is the most convenient of all mutagens to use and it is also very easy to take effective safety precautions against it. The UV light is the best studied mutagenic agent in prokaryotic organisms. It gives a high proposition of pyrimidine dimers and includes all types of base pair substitutions (Meenu et al., 2000).

N-Methyl-N'-nitro-N-nitrosoguanidine(NTG) has been widely used to induce mutations in bacteria. It has proved highly effective, so much so that it has been suggested to be the most potent chemical mutagen yet discovered (Adelberg, Mandel & Chen, 1965). Adelberg et al**.** (1965) found that mutations to valine resistance and to auxotrophy occurred at high frequency (up to 42-5 % auxotrophs) after exposure of *Escherichia coli* to NTG under conditions such that about 5 % of the treated bacteria remained viable. NTG was also proved to be a very effective mutagen for yeasts, though the results are less dramatic than with *E. coli***.** Ellaiah P (1986) with *Schizosaccharomyces pombe* found that, without selection, NTG-induced auxotrophs increased to a maximum frequency of about 8 % at 20 % survival. On the other hand, Nordstrom (1967) obtained up to 50 % petite mutants among survivors of *Saccharomyces cerevisiae* after NTG treatment.

MATERIALS AND METHODS

Chemicals

All chemicals and medium constituents used for the present study were procured from M/S Hi-media, Mumbai.

Microorganism

The newly isolated bacterial species i.e. *Bacillus cereus* MS-6 strain (wild strain) that produce L-asparaginase was employed in the present study. The selected isolate was grown on M-7 agar slants at 37oC for 24 h, subcultured at monthly intervals and stored in the refrigerator.

Preparation of Inoculum

Inoculum was prepared as per the general procedure. (M. Sunitha et al, 2010)

Shake flask fermentation

The fermentation was conducted as per the general procedure. (M. Sunitha et al, 2010)

Analytical method

First step in the analytical method is UV irradiation of parent strain and selection of mutants (Hopwood et al., 1985). Figure 1 and 2

The next step is NTG treatment and selection of mutants (Cerda-Olmedo and Hanawalt, 1968; Adelberg *et al*., 1965)., (Cerda-Olmedo and Hanawalt, 1968). Table 1 and fig 3. then the mutants are treated with EMS (M. Sunitha et al 2010). Table 2 and fig 4.

a) UV irradiation of parent strain and selection of mutants

Strain improvement for the selected parent strain was done by mutation and selection. The wild strain (MS-6) was subjected to UV irradiation. The dose survival curve was plotted for selecting the mutants between 10 and 0.1% rate. Mutation frequency was mentioned to be high when the survival rates were between 10 and 0.1% (Hopwood et al., 1985).

b) NTG treatment and selection of mutants

The best UV-mutant of *Bacillus cereus* MUV-9 was selected for NTG treatment. The NTG (150 mg) was accurately weighed and dissolved in 25 ml of phosphate buffer (pH 8.5) at a temperature of 4ºC to minimize decomposition and sterilized by passing through sterile bacterial filter (0.22 µm).

NTG is considered to be a very effective mutagen (Cerda-Olmedo and Hanawalt, 1968; Adelberg *et al*., 1965). Its action has been attributed to its decomposition products (Cerda-Olmedo and Hanawalt, 1968). The treated samples were diluted, plated and colony counts were made. The percentage survival or kill was calculated and data is presented in Table 4.2. The survival curve was plotted (Fig. 4.3). Selected colonies (12) were picked up from 120 and 180 min. exposure and transferred on to M-7 medium agar slants. The slants were incubated at

37^0C for 24 h to obtain maximum growth. They were labeled as MNTG 1 to MNTG 12.

c) EMS treatment and selection of mutants

The highest producing MNTG mutant (**MNTG-7**) was selected for EMS treatment. The cell suspension of MNTG-07 was prepared as described earlier and the cell pellets obtained after centrifugation were resuspended in phosphate buffer (pH 8.0).

Five ml of each cell suspension was added to 15 ml of EMS solution (4 mg/ml) and incubated at 37°C (Akhund and Khvostova, 1966). Two ml of this solution was taken at intervals of 0, 30, 60, 90, 120, 150, 180, 210, and 270 min. and centrifuged immediately. The supernatant was decanted and the cell pellet obtained was resuspended in 5 ml of sterile distilled water to stop the reaction. The total time from the first incubation of the cell suspension to re-suspension into distilled water was recorded.

RESULTS

From the results (Fig. 2), it is evident that UV induced mutant MUV-9 showed highest L-asparaginase activity (24.98 IU/ml). It was 3.059 fold improvements over the parent strain.

Further mutagenesis of this strain by NTG treatment could yield few mutants. Examination of L-asparaginase activity of the selected mutants indicated that the mutant MNTG-7 has further improvement in L-asparaginase activity (38.06 IU/ml) (Fig. 5). It was 4.66 fold improvement in yield over the wild strain (MS-6).

DISCUSSION

The strain of *Bacillus cereus* MS-6 was subjected to strain improvement program with a view to obtain increased L-asparaginase production. The most effective mutagens, Ultra Violet rays (UV) and N methyl-N'-nitro-Nnitrosoguanidine (NTG) were chosen for strain improvement. The results indicated that among UV mutants, MUV-9 showed the highest L-asparaginsase activity (24.98 IU/mL). it was 3.059 fold of the parent strain. The chemical mutagen (NTG) yielded a better enzyme producing mutant MNTG-7 with 38.06 IU/mL. Thus the strain improvement programme resulted in a mutant that produced 12.11 fold higher amount of the enzyme production over the parent wild strain (3.14 IU/mL), which is a very significant achievement in enzyme yield.

TABLES AND FIGURES

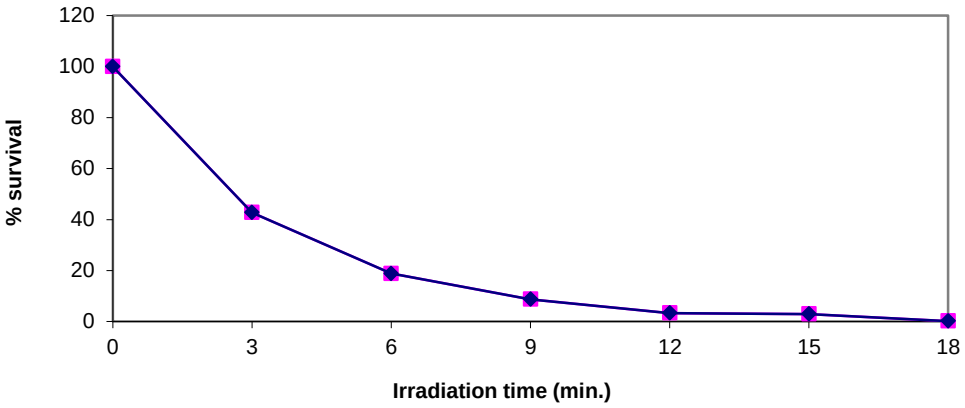

Fig. 1: Survival curve of isolate MS-06 after UV irradiation

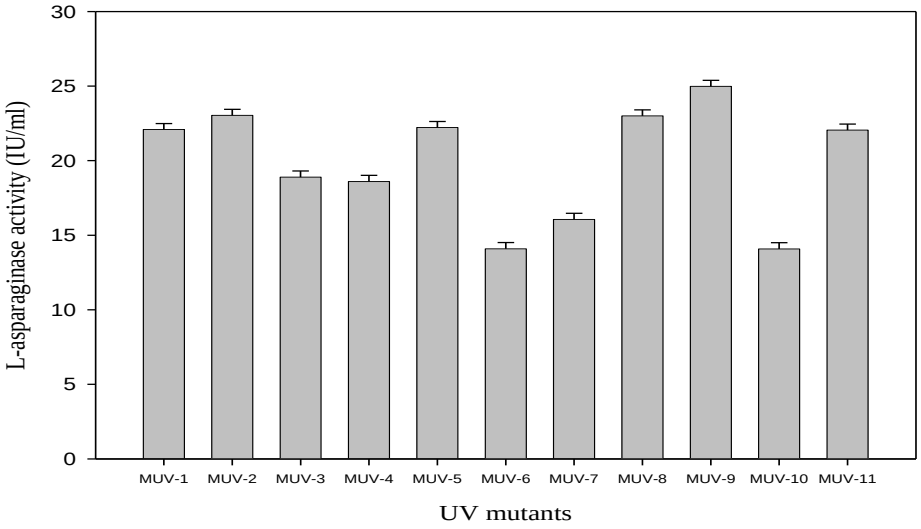

Fig. 4.2: Production of L-asparaginase by UV mutants

Table 1: Effect of NTG on MUV-9

Exposure time (min)	Number of cells/ml after treatment	Percentage kill	Survival percent
0	4.4×10^7	0	100
30	4.1×10^7	6.82	93.18
60	2.9×10^7	34.09	65.91
90	6.8×10^6	84.55	15.45
120	6.1×10^6	86.14	13.86
150	7.9×10^5	98.2	1.8
180	4.9×10^5	98.89	1.11
210	4.43×10^5	98.99	1.01
270	3.2×10^5	99.27	0.73

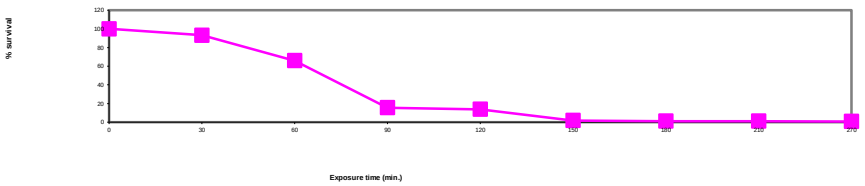

Fig. 3: Survival curve of isolate MUV-9 after NTG treatment

Table 2. Effect of EMS on MNTG-07

Exposure time (min)	Number of cells/ml after experiment	Percentage kill	Survival percent
0	20×10^{7}	0	100
30	10×10^{7}	50	50
60	60×10^{6}	70	30
90	23.3×10^{6}	88.35	11.65
120	20×10^{6}	90	10
150	16×10^{6}	92	8
180	14×10^{6}	93	7
210	12×10^{6}	94	6
270	8×10^{5}	99.6	0.4

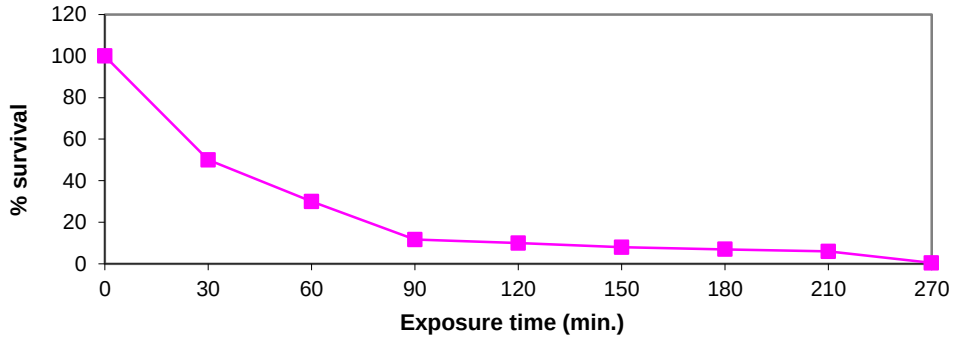

Fig.4. Survival curve of isolate MNTG-7 after EMS treatment

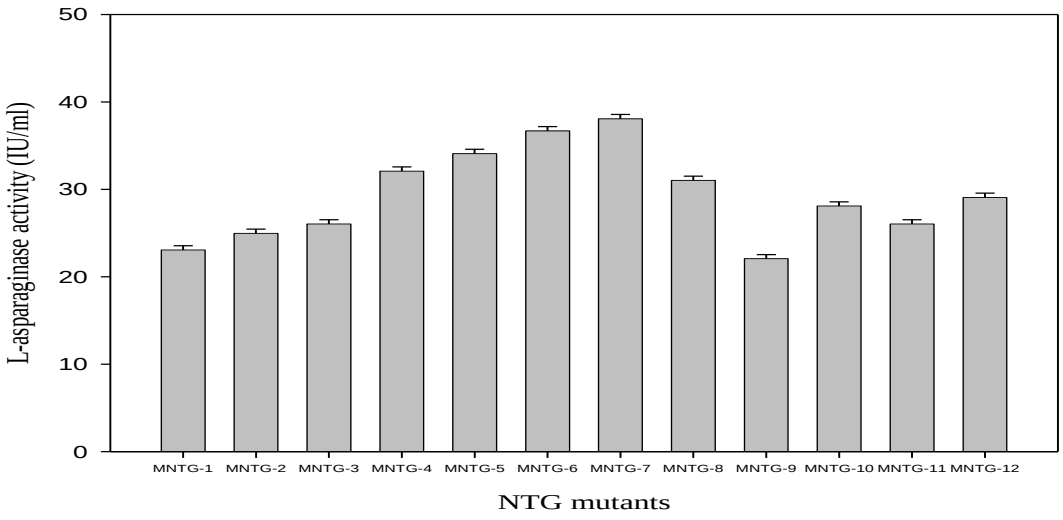

Fig. 4.4: L-asparaginase production by NTG mutants

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