STRAIN IMPROVEMENT STUDIES

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,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.

The methods available in applied genetics involve trial and error. Strain development programs generally involve the following stages: 1) induction of genetic variation, 2) pre-selection, 3) screening of selected strain in shake flask, followed by tests in laboratory fermentor, 4) evaluation of selected strain at pilot plant scale and 5) introduction on a production scale in the main plant.

Mutagenesis

The classical genetic approach to improve the metabolite yield is to subject the organism to random mutations using various mutagenic agents and then to screen the survivors after these lethal treatments for colonies that show increased enzyme production. This process would be repeated until no further increase could be detected.

Choice of mutagen

While selecting mutagen for use in strain improvement program, one should consider the phenomenon of mutagen specificity, where by a given mutagen or mutagenic

treatment preferentially mutates certain parts of the genome, while unaffecting the other parts. 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 (UV) 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).

The EMS is also used for strain improvement as it induces linked multiple mutations at fairly high frequencies. It promotes base pair substitutions, primarily GC-AT transitions.

Dosage of mutagen

The optimum concentration of mutagen is that which gives the highest proportion of desirable mutants in the surviving population. Hopwood et al. (1985) suggested that 99.9% kill is best suited for strain improvement programs as the fewer survivors in the treated sample would have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the metabolite.

Two important conventional techniques viz., UV irradiation and HNO_2 treatment have been carried out for yield improvement studies in this work..

Experimental

Chemicals

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

Microorganisms

Isolated GAS-4(wild strains), that produces protease, was employed in the present study. These organisms were isolated from soil samples from Andhra Pradesh State, India. The isolates were grown on starch casein agar slants at 28°C for 96 h, subcultured at monthly intervals and stored in the refrigerator.

Preparation of spore suspension

Each organism grown on starch casein agar slant was scraped off into sterile water containing tween-80 (1:4000) to give a uniform suspension. The suspension was transferred into sterile conical flasks (250ml) containing sterile glass beads and thoroughly shaken for 30 min. on a rotary shaker to break the spore chains. The spore suspension was then filtered through a thin sterile cotton wad into a sterile tube, to eliminate vegetative cells from the suspension, so that after plating each spore was germinated to give a colony. The spore suspension was diluted and used for plating.

Shake flask fermentation

Five ml of inoculum (10% v/v) was added to the 45 ml of production medium in 250 ml Erlenmeyer flask. The flasks were incubated at 28°C on rotary incubator shaker for 96 h (180 rpm with 5 cm through). At the end of fermentation, 5ml broth was collected and centrifuged at 3000 rpm for 10 min. and assayed for protease activity. The composition of production medium is: g/100 ml Glucose-0.5, yeast extract – 0.25, Tryptone – 0.25, pH-7.0.

Analytical method

The protease activity was determined by Lowry method as described earlier.

Mutation and selection

a) UV Irradiation of parent strain and selection of mutants

Strain improvement for the selected parent strain were done by mutation and selection. The wild strain (GAS-4) 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).

The spore suspension of wild strains was prepared in phosphate buffer, pH 8.0 and 4 ml quantities were pipetted aseptically into sterile flat bottomed petridishes of 100 mm dia. The exposure to UV light was carried out in a "Dispensing - Cabinet" fitted with TUP 40W Germicidal lamp that has about 90% of its radiation at 2540-2550A. The exposure was carried out at a distance of 26.5 cm away from the center of the germicidal lamp. The exposure was carried out for 0, 30, 60, 9, 120, 180 and 240 seconds respectively. During the exposure, the lid of the Petridis was removed. Hands were covered with gloves and the plates were gently rotated so as to get uniform exposure of the contents of the Petridis. During the treatment, all the other sources of light were cut off and the exposure was carried out in dark (during night time). The treated spore suspensions were transferred into sterile test tubes covered with a black paper and kept in the refrigerator overnight, to avoid photo reactivation.

Each irradiated spore suspension was serially diluted with sterile phosphate buffer solution (PBS). The spore suspensions after suitably diluting in the buffer (PBS) with pH -7 were plated onto starch casein agar medium and incubated for 96 h at 28°C. The number of colonies in each plate was counted. It was assumed that each colony was formed from a single spore. The number of survivals from each exposure time for GAS-4are represented

in Table 4.1. The UV survival curves are plotted (Fig. 4.1). Plates having less than 1% survival rate (60 and 120 sec.) were selected for the isolation of mutants. The isolates were selected on the basis of macroscopic differential characteristics. The selected isolates were subjected to fermentation and tested for their alkaline protease production capacities as described earlier (Fig. 4.3). The best protease producing UV mutant strain (UV A8) was selected for HNO_2 treatment.

b) Nitrous acid treatment:

The cell suspension of the strain UV A8 was prepared by using Acetate buffer pH 7.0. To 9mL of the cell suspension in buffer 1mL sterile stock solution of 0.01 M Sodium Nitrite was added. Samples of 4mL were withdrawn at, 30, 60, 120, 180, 240 seconds respectively. Each of 1mL sample was neutralized with 0.5mL of 0.1M NaOH. Serially diluted and plated on the YEME medium.

Plates having survival rate between 15 and 1% were selected for the isolation of mutants. The stable mutants UV A1 to UV A13 were selected based on the consistent expression of the phenotypic characters and maintained on YEME slants. The plates were incubated at 28^0C for 5 days.

RESULTS AND DISCUSSION:

Genetic improvement is one of the promising approaches for increased production of enzymes by industrially important microorganisms. Genetic improvement of the selected GAS-4 strain was carried out by physical and chemical mutagenesis. In the present investigation, mutations were induced physically by using UV irradiation and chemically by Nitrous acid treatment.

A. UV irradiation of parent strain GAS-4 and selection of mutants:

The strain was subjected to UV Irradiation. Mutation frequency was observed to be high when the survival rates were between 27 and 1%..

The dose survival curve was plotted and presented in Table 4.1.

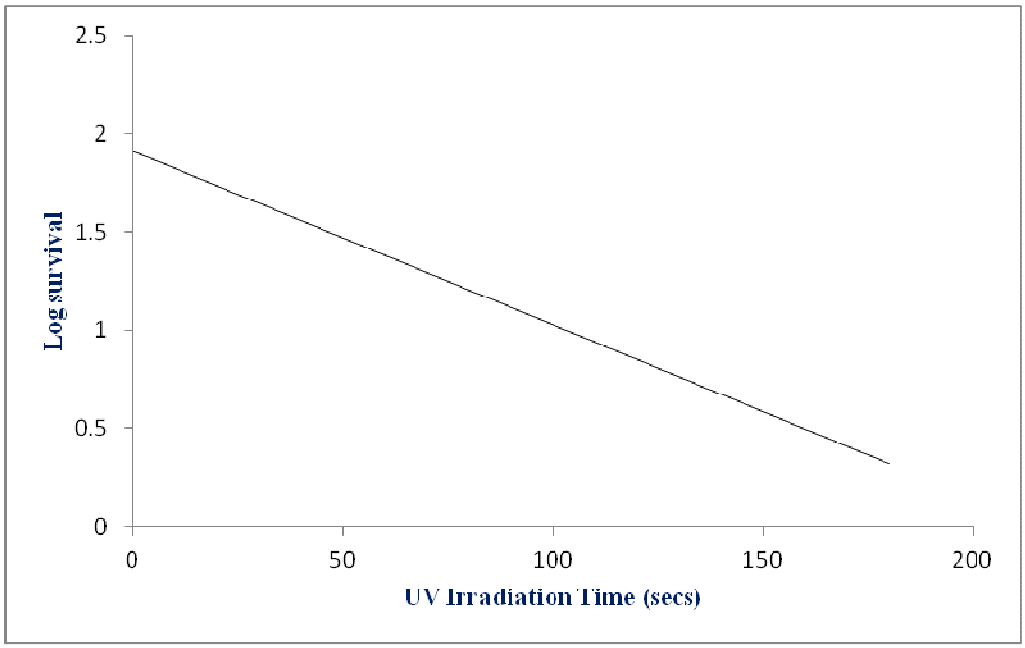
Table. 4.1 Effect of UV Irradiations on Isolate GAS-4

S.No	Irradiation Time (secs)	Number of colonies/mL after irradiation ($x10^5$)	Percentage Kill (%)	Survival Percentage (%)	Log Survival
1.	0	162	0	100	2
2.	30	91	43.82	56.18	1.74
3.	60	23	85.80	14.20	1.15
4.	120	11	93.20	6.80	0.83
5.	180	0.0004	97.53	2.46	0.39
6.	240	0	100	0	0

UV Survival Curve:

UV Survival Curve is plotted by taking UV Irradiation time on X-axis and log survival on Y-axis. (Fig 4.1)

Fig 4.1 UV Survival Curve of the isolate GAS-4

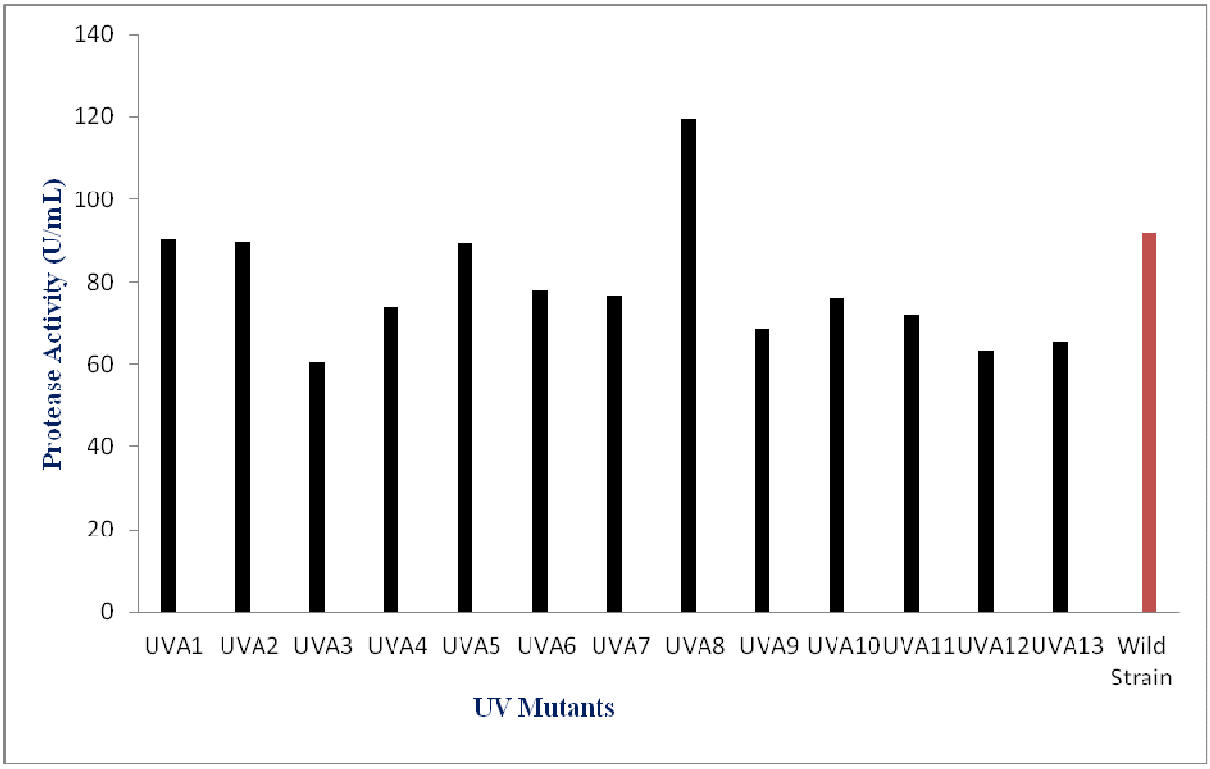


Protease activity of the UV mutants: A total 13 mutants were isolated and their Protease production capacity by submerged fermentation was determined according to the Lowry method (Lowry et al. 1951).

Table. 4.2: Protease activity of UV mutants

S.No	UV Mutants	Protease activity (U/ml)
1	UVA1	90.6
2	UVA2	89.6
3	UVA3	60.5
4	UVA4	73.9
5	UVA5	89.3
6	UVA6	78.2
7	UVA7	76.5
8	UVA8	119.4
9	UVA9	68.7
10	UVA10	75.9
11	UVA11	72.0
12	UVA12	63.2
13	UVA13	65.4
14	Wild Strain	92.0

Fig 4.2 Protease Activity of UV Mutants of the isolate GAS-4



- Out of UV 13 mutants
    - UVA8 showed a maximum Protease activity of **119.4U/ml** while the wild strain showed 92U/ml.(Table. 4.2) & (Fig. 4.2)
    - Production of Protease by UVA8 mutant was **27.4 %** higher than the parent wild strain (GAS-4).
- Hence UVA8 was selected for subsequent Nitrous acid treatment.

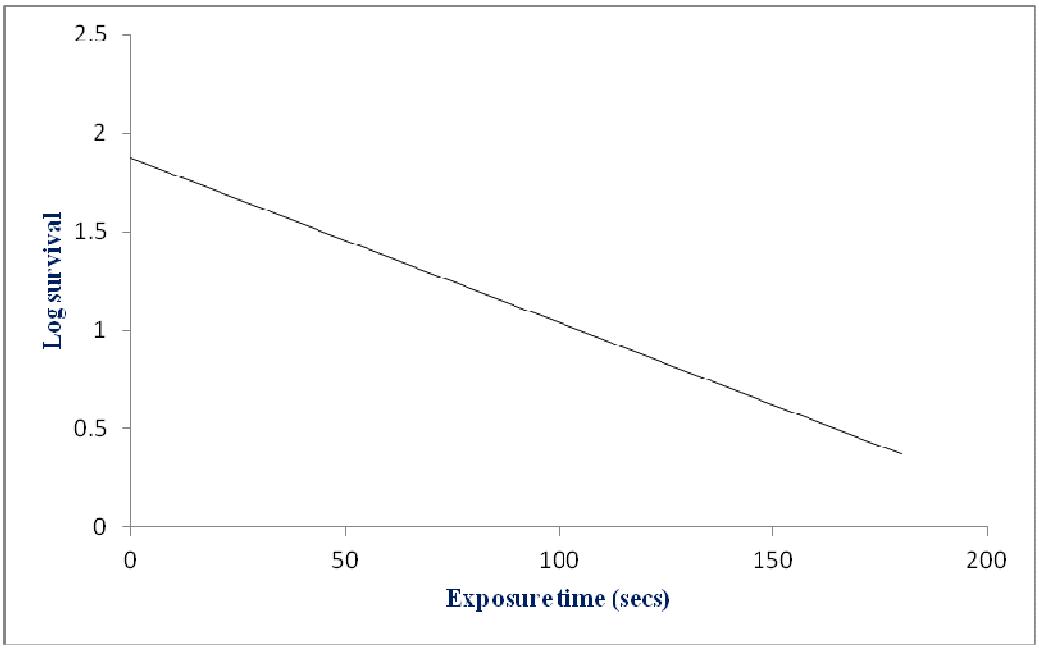
B. Nitrous acid treatment of UV-8 mutant:

The selected mutant UVA8 was subjected to nitrous acid treatment. The dose survival curve was plotted and presented in Table 4.3 and Fig 4.3.The survival curve was plotted by taking Exposure time on X-axis and % survival on Y-axis. (Fig 4.3)

Table. 4.3: Effect of Nitrous acid on UVA8 Mutant

S.No	Irradiation Time (secs)	Number of colonies/mL after irradiation ($x10^5$)	Percentage Kill (%)	Survival Percentage (%)	Log Survival
1.	0	156	0	100	2
2.	30	87	44.23	55.7	1.74
3.	60	18	88.46	11.54	1.06
4.	120	10	93.58	6.42	0.80
5.	180	0.0005	96.79	3.20	0.50
6.	240	0	100	0	0

Fig. 4.3 Survival Curve of UVA8 mutants after Nitrous acid treatment.



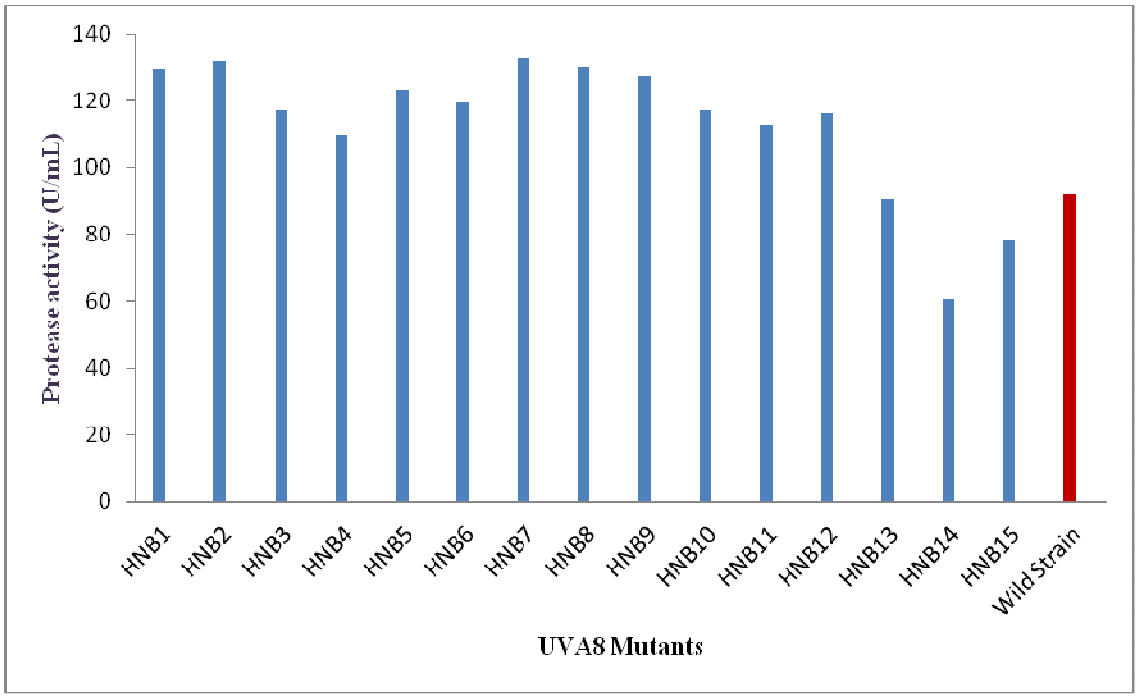
Protease activity by various Nitrous acid mutants of UVA8:

A total of 15 mutants were selected and determined for their Protease production capacities by submerged fermentation and the activity was determined according to Lowry method (Lowry et al. 1951).

Table. 4.4: Protease Activity by various Nitrous acid mutants of UVA8

S.No	UVA8 mutants	Protease activity (U/ml)
1	HNB1	129.6
2	HNB2	131.7
3	HNB3	117.2
4	HNB4	109.8
5	HNB5	123.1
6	HNB6	119.6
7	HNB7	132.5
8	HNB8	130.1
9	HNB9	127.2
10	HNB10	117.2
11	HNB11	112.9
12	HNB12	116.2
13	HNB13	90.4
14	HNB14	60.7
15	HNB15	78.6
16	Wild Strain	92.0

Fig. 4.4: Protease activity by various Nitrous acid mutants of UVA8



Out of 15 Nitrous Acid mutants

- **HNB7** showed maximum Protease activity of 132.5U/ml, which was **12.6** times more than the mutant strain UVA8 (119.4 U/ml). (Table 4.4) & (Fig. 4.4)
- Compared to the wild strain (80 U/ml) the mutant HNB7 produced 132.5 U/ml. This represents an increase of **60.6 %** compared to the wild strain.

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