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SCREENING OF *BACILLUS LICHENIFORMIS* MUTANTS FOR IMPROVED PRODUCTION OF ALPHA AMYLASE

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Abstract

The present study is concerned with the screening of hyper -secretive mutant of *Bacillus licheniformis* for the production of alpha amylase. The parental strain of the *Bacillus licheniformis* was subjected to UV irradiations for 5-30 minutes. Among 257 mutants isolated, the mutant GCUM-15 gave maximum production of alpha amylase (416 IU/ml/min). This mutant was further subjected to UV irradiation for 25 minutes. Among 18 mutants tested for enzyme production, the mutant GCBU-8 was found to be more potent for enzyme production (524 IU/ml/min). This mutant was further subjected to NTG treatment for 5-60 minutes. The amount of NTG 200 mg/ml for 30min was found to be best for the production of stable and viable mutants. The mutant GCCM-23, better producer of enzyme was again treated alternatively with UV/NTG and the best alpha amylase secretive mutant *Bacillus licheniformis*GCUCM-30 was selected. This mutant gave 12 times more enzyme (788 IU/ml/min) than the original parental strain. When this mutant was again treated with UV or chemically, complete death of the bacteria was observed. The kinetic parametric study indicated that the yield of the enzyme was ten times high by this mutant than the parental strain. The volumetric rates of biomass formation and substrate consumption were also high by the selected mutant and the mutant entered in the stationary phase earlier than the parental strain.

Introduction

Alpha amylase finds commercial applications in food, textile, pharmaceuticals and detergent industries (Prescott and Dunn 1987). Highly active alpha amylase from *Bacillus* is very essential to meet the demand of above-mentioned industries (Yuguo *et al.*, 1993; Pedersen and Nielsen, 2000). The mutant strain of *Bacillus* has better ability to produce alpha amylase, which can be derived by mutagensis and extensive screening (Zhao and Qirong 1994). Both chemical mutagenic agents as well as UV irradiations can be used to improve the *Bacillus* strains for the hyper production of alpha amylase (Xuezhi *et al.*, 1991). The N-methyl-N-nitro-N-nitroso guanidine (NTG) was found to be better mutagenic agent for the production of mutants of *Bacillus* as well as for the production of alpha amylase (Eugenia *et al.*, 1992). The present study is concerned with the selection of best mutant of *Bacillus licheniformis,* isolated and selected (Haq *et al*, 1997), for the production of alpha amylase after exposing the bacteria to UV and then treated with NTG.

Materials and methods

Organism: The parental strain of *Bacillus licheniformis* GCB-36 was obtained from Biotechnology Laboratory, Department of Botany, Govt. College, Lahore. The strain was maintained on the nutrients starch agar slopes.

Fermentation technique: Fifty ml of the fermentation medium containing (g/L) nutrient broth 20, starch 10.0, lactose 10.0, NaCl 2.0 $CaCl_2$ 2.0 in 1000-ml phosphate buffer (pH 7.5) was transferred to 250ml of cotton plugged conical flask.

All the experiments were run in triplicates. The flasks were sterilized in the autoclave at 15-lb/inch^2 pressure at 121°C for 15 minutes. The flasks were cooled at room temperature and each flask was inoculated with one ml of inoculum. The flasks were then rotated in the rotary incubator shaker (200-rpm) at 40°C for 48 hours. After 48 hours, the contents of the flasks were centrifuged at 5000 rpm and substrate free supernatant was used for the estimation of alpha amylase.

Mutagenic treatments

Preparation of cells for mutagenic treatment: Twenty-four hours old bacterial culture prepared in nutrient broth medium was centrifuged aseptically for 10 minutes. The bacterial cells were then suspended in 50 ml of saline water. The suspension was then diluted up to 10^{-3} to 10^{-5} times.

Ultraviolet treatment: Ten ml of above diluted suspension was transferred to the sterilized petriplates. The petriplates were then placed under the UV lamp, (emittingthe energy of 1.6 x 10^2 J/m^2/s) for 5-30 minutes. After the time intervals, 0.5 ml of the bacterial suspension was transferred to the petriplates containing nutrient starch agar medium. The plates were than placed in the incubator at 40°C for 24 hours. After 24 hours, the bacterial colonies with bigger zones of hydrolyzing starch as compared to parental strain (which was also run in parallel) were picked up and then transferred to nutrient starch agar slants.

Chemical treatment: Five ml of prepared (50-300mg/ml) N-methyl-N-nitro-N-nitroso guanidine (NTG) was transferred to each sterilized centrifuged tube containing 5 ml of bacterial suspension. The tubes were placed at room temperature for different time intervals such as 5-60 minutes. After the time interval, the tubes were centrifuged at 7000 rpm for 30 minutes. The supernatant was discarded to remove the NTG from the bacterial cells. Ten ml of saline water was added to each centrifuged tube. The tubes were re-centrifuged for the removal of traces of NTG from bacterial cells. This process was repeated three times. After washing the cells, 10ml of sterilized saline water was added to each tube to form bacterial suspension. 0.2 ml of this suspension was transferred to the petriplates containing nutrient starch agar plates and incubated at 40°C for 24 hours. After 24 hours, the bacterial colonies showing bigger zone of hydrolyzing starch as compared to parental strain were picked up and transferred to the nutrient starch agar slants.

Enzyme assay Alpha amylase estimation was carried out according to the method of Rick and Stegbauer (1974). The enzyme solution at pH 7.5 was incubated at 60°C using 1% soluble starch solution. The reducing sugars were measured by adding 3,5-dinitro salicylic acid reagent, boiling for 5 min, cooling and measuring the O.D at 540 nm in the spectrophotometer (Model CECIL CE7200) against maltose as standard. One unit of activity is equivalent to that amount of enzymes, which in 10 minutes librates reducing group from 1% Lintner's soluble starch corresponding to 1 mg maltose hydrate.

The amylase activity was determined in IU/ml/min by applying the following formula

$$\text{IU/ml/min} = \frac{\text{Activity of enzyme x 1000}}{\text{Molecular wt of maltose x time of incubation}}$$

Biomass: The biomass was determined turbidimeterically at 650nm in spectrophotometer and read against graph plotted for dry weight Vs. O.D. at the same wavelength. The biomass was expressed as g/l (Hamilton *et al*, 1999).

Kinetic study& Statistical analysis: Kinetic parameters for batch fermentation were determined after Pirt (1975). Treatment effects were compared by the method of Snedecor and Cochrane (1980). Significance has been presented as Duncan multiple range tests in the form of probability (*P*) values.

Results & Discussion

The mutant strains of *Bacillus* species have better ability to produce alpha-amylase (Allan *et al.*, 1996; Allan *et al.*, 1997). For the improvement of the bacterium, vegetative cells of *Bacillus licheniformis* were subjected to UV irradiations for 5-35 min (Table 1). Two hundred and fifty seven mutants were isolated by observing bigger zones of hydrolysis as of starch in the petriplates as compared to parental strain. Of all the mutant strain tested, the *Bacillus licheniformis* GCBU-25, isolated after 25 min of UV irritation, gave maximum production of alpha amylase (416 IU/ml/min). The improvement in the enzyme formation may be due to the photolysis of pyramidines, in adjacent pyramidines to form dimmers. They may cause error at the next replication and so result in mutation. The gene responsible for the production of alpha amylase may increase on the DNA of bacteria due to mutation, as a result increase in enzyme production (Markkanen and Suihko 1974; Gardner *et al.*, 1991). Further increase in the UV treatment, resulted in the complete death of bacteria. Thus exposure time of 25 minutes was selected because it gave enhancement of alpha amylase formation. This mutant strain was again subjected to UV irradiation for further improvement (Table 2). Eighteen mutants were selected and the mutant no. 8 gave the maximum production of alpha amylase (524 IU/ml/min). This mutant was assigned the code as GCBU-8.

The UV treated mutant strain was further treated with different doses of NTG for 5-60 minutes (Table 3 & 4). Four hundred and thirty mutants were isolated by observing the bigger zones of hydrolysis of starch in the petriplates as compared with the UV selected strain (Akpan *et al* 1999). These mutants were then evaluated for alpha amylase production. Among all the mutants tested, mutant isolated after 30 minutes of NTG (200mg/ml) treatment was found to be most potent for alpha amylase production (654 IU/ml/min). The complete death of bacteria was observed when the treatment time was increased from 50 minutes or with higher dose of NTG. This mutant was assigned the code as GCCM-23.

To obtain the hyper producer of alpha amylase, the mutant GCCM-30 was again treated alternatively with UV and NTG (Table 5) Thirty mutants were isolated after first alternate treatment and the amount of alpha amylase production was ranged from 580-700 IU/ml/min. The mutant that produced maximum production of enzyme (700 IU/ml/min) was again given alternate treatment. Among the 25 mutants isolated and tested for enzyme production, only one mutant gave best production of alpha amylase (788 IU/ml/min). As this mutant was again treated alternatively no further improvement was observed. It may be due to the application of mutagenic agent to a wild type strain; better initial improvement can be expected,

followed by plateau effect. This plateau represents the saturation value of positive mutant that can be induced in the site of attack of the mutagen by same mutagen. A second mutagen may attack the same site in a different way or may have a different site of attack. A second phase of strain improvement can be expected followed by plateau. Thus, no further improvement was found after treatment with NTG. This finding is an agreements with the work reported by Baily and Markkanen (1975). However, the mutant, that gave best production of alpha amylase, was assigned the code as *Bacillus licheniformis* GCUCM-30. This mutant produced 12 times more alpha amylase than the original parental strain.

The parental and mutant derivatives were compared for the production of alpha amylase (Fig 1&2). It is clear from the figs that the there was gradual increase in the cell mass and enzyme formation as the mutants were derived from one another. The production of enzyme was reached maximum 48 h after inoculation by the parental, UV mutant and NTG derived mutant. However, the alternate treated mutant gave maximum production of enzyme 44h after inoculation. It might be due to this mutant reached in the stationary phase earlier than the other strains and accumulation of alpha amylase was maximum at that time (Lealem and Gashe 1994). The kinetic parametric values (Fig 3) such as Yp/x and Yp/s indicated that the yield of the enzyme by *Bacillus licheniformis* GCUCM-30 was ten times greater than the original parental strain. The values of Qp, Qs, Qx (Table 6) also show significance over the other strains for the production of alpha amylase.

The present study is found to be quite beneficial because by using mutagenic agents hyper producer mutant of *Bacillus licheniformis* was achieved. It leads towards savings in the economy of the country.

Table 1: Screening of *Bacillus* mutants for the production of alpha amylase isolated after different time intervals of UV irradiations.

No.	Exposure Time (min)	No. of Survivals	Range of Alpha-amylase IU/ml/min
1	5	90	13 ±2 - 291±3
2	10	60	34 ±1 - 367±4
3	15	55	34±2 - 389±3
4	20	32	106±1 - 370±1
5	25	20	223±5 – 416±2
6	30	Nil	31±4 - 361±6
7	35	Nil	Nil

Each value is an average of three replicates. ± indicated the standard deviation from mean value.

Table2: Screening of *Bacillus* mutants after further UV treatment.

No.	Survivals	IU/ml/min
1	GCUM1	471±3
2	GCUM2	432±4
3	GCUM3	463±2
4	GCUM4	324±1
5	GCUM5	471±3
6	GCUM6	416±5
7	GCUM7	337±2
8	GCUM8	524±3
9	GCUM9	333±1
10	GCUM10	322±5
11	GCUM11	393±2
12	GCUM12	458±1
13	GCUM13	431±1
14	GCUM14	340±4
15	GCUM15	380±3
16	GCUM16	458±2
17	GCUM17	267±2
18	GCUM18	200±1

Each value is an average of three replicates. ± indicated the standard deviation from mean value.

Table3: Screening of *Bacillus* mutants after different doses of NTG.

No	NTG concentrations (mg/ml)	No of survivals	Range of alpha amylase IU/ml/min
1	50	78	20±3 - 81±2
2	100	64	100±1 - 225±4
3	150	32	135±6 - 510±2
4	200	17	320±3 - 600±2
5	250	12	124±2 - 470±2
6	300	Nil	Nil

Each value is an average of three replicates. ± indicated the standard deviation from mean value.

Table 4: Screening of NTG treated mutants isolated after different intervals of time.

No.	Treatment Time (min)	*No. Of survivals*	Range of Alpha amylase (IU/ml/min)
1	5	62	29±3 - 317±5
2	10	49	53±1 - 506±4
3	15	35	53±2 - 563±4
4	20	20	327±4 - 476±4
5	25	17	262±2 - 527±3
6	30	11	393±2 - 654±1
7	35	12	230±3 - 602±3
8	40	08	231±1 - 530±0.5
9	45	08	130±4 - 526±5
10	50	05	380±6 - 471±1
11	55	NIL	NIL
12	60	NIL	NIL

Each value is an average of three replicates. ± indicated the standard deviation from mean value.

Table 5: Screening of Mutants After Alternate Treatments of UV and NTG

No	No of Treatments	Survivals	Range of alpha amylase (IU/ml/min)
1	First	30	580±3 - 700±6
2	Second	25	700±2 - 788±2.5
3	Third	8	610±5 - 690±4
4.	Fourth	Nil	Nil

Each value is an average of three replicates. ± indicated the standard deviation from mean value.

Table 6: kinetic parameters for the production of alpha amylase following growth of mutant and parental strain of *Bacillus licheniformis*.

Kinetic parameters	Parental strain	UV Mutant	NTG Mutant	UV/NTG Mutant
Yx/s (g/g)	0.84±0.02	0.86 ±0.02	0.90 ±0.02	0.90 ±0.02
Qp (IU/ml/h)	2.0±0.12	5.4 ±0.01	12.1 ±0.02	14.7 ±0.01
Qs (g/l/h)	0.4±0.01	0.4±0.03	0.54 ±0.04	0.56 ±0.03
Qx (g/l/h)	0.4 ±0.02	0.4 ±0.03	0.4 ±0.018	0.4±0.019

Each value is the average of three replicates

Fig 1 Comparison between Parental strain of *Bacillus licheniformis* and its mutants derivatives for the production of alpha amylase

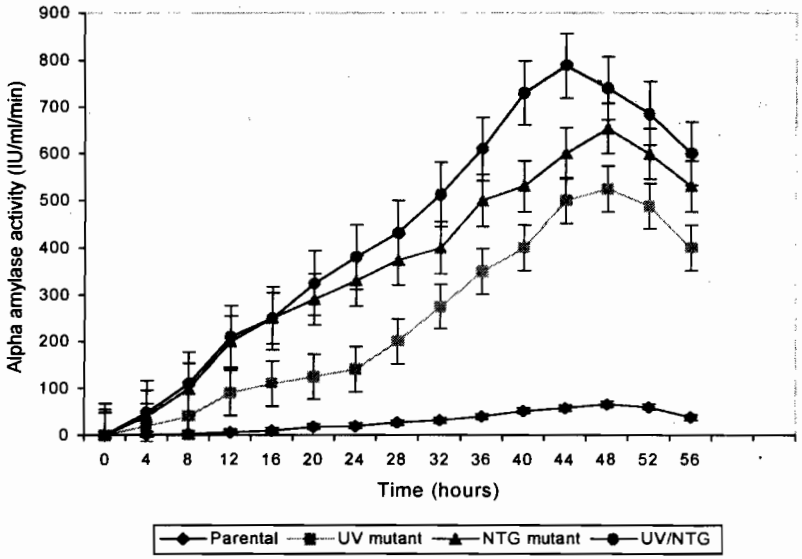
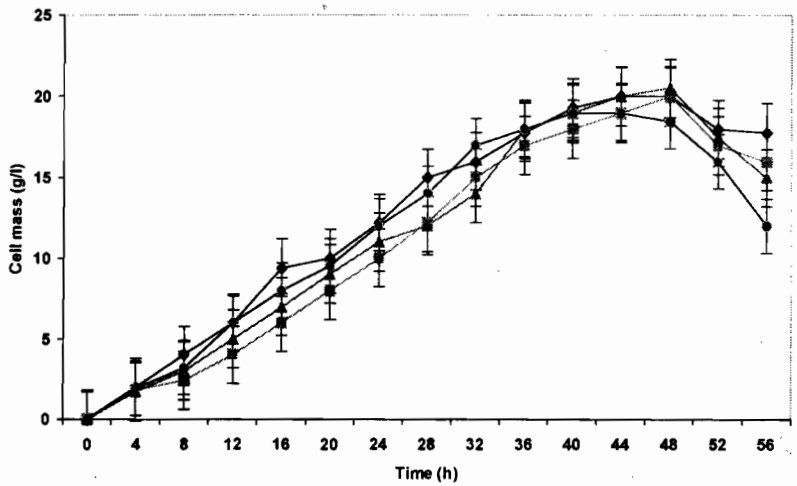
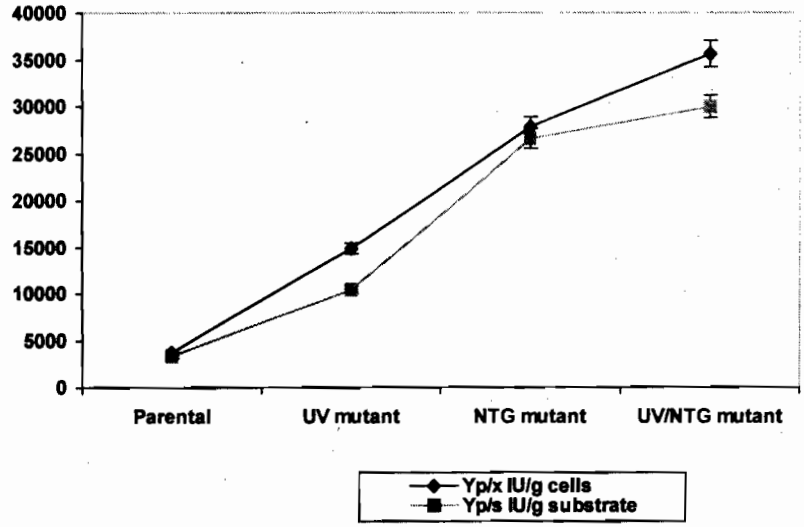


Fig 2 Comparison between *Bacillus* strains for the production of cell mass.



Each value is an average of three replicates. Y error bars indicated the standard error from mean value. The values differ significantly at $P < 0.05$

Fig3 Kinetically comparison between the parent and its mutant Derivatives for the yield of alpha amylase.



Each value is an average of three replicates. Y error bars indicated the standard error from mean value. The values differ significantly at $P < 0.05$

References

Akpan,I., Mankole, M.O and Adesemowo, A.M. 1999. A rapid plate culture method for screening of alpha amylase producing microorganisms. *Biotechnol. Techniques*, 13(6): 411-413.

Allan, S., B. Torbenvedel and B.F. Henrick. 1997. Recombinent alpha amylase mutants and their use in textile desizing, starch liquification and washing. *PTC.Int. Appl.*, 12(5): 205-210.

Allan, S., B.F. Henrick and B. Torbenvedel. 1996. A method of designing alpha amylase mutants, with predetermined properties, alpha amylase variants and detergents contaning the variants. *Process Biochem.*, 31(5): 110-210.

Bailey, M. J. and P. H. Markkanen. 1975. Use of mutagenic agents in Improvement of α-amylase Production by *Bacillus subtilis J. Appl. Chem. Biotechnol.* 25: 73-79.

Eugenia, D., M. Doina and R. O. Rom. 1992. *Bacillus subtilis* mutant with high production potential for amylolytic enzyme. *Mencenicopschi Gheorgh*, 101: 113.

Hamilton, L.M., W. M. Fogarty and C. T. Kelly. 1999. Purification and properties of the raw starch degrading alpha amylase of *Bacillus* sp. IMD-434. *Biotechnol, Lett.*, 21:111-115.

Haq, I., H. Ashraf, S. Ali, and M. A. Qadeer,. 1997. Submerged fermentation of alpha amylase by *Bacillus licheniformis* GCB-36. *Biologia* , 43(2):39-45.

Gardner, J.E., J. E. Simmons and D.P. Snustad. 1991. *Principal of Genetic*. 8th Edition. Jhon Wiley and Sons, Inc. 304-305.

Lealem, F. and B. A. Gashe. 1994. Amylase production by a gram positive bacterium isolated from fermenting *J. Appl. Bacteriol.*, 77(3): 348-352.

Markkanen, P.H. and M.L. Suihko. 1974. The use of UV radiation in the improvement of enzyme production by *Bacillus subtilis Fina. Chem. Lett.* 2: 89-92.

Pedersen, H. and J. Nielsen. 2000. The influence of nitrogen sources on the alpha amylase productivity of *Aspergillus oryzae* in continuous cultures. *Applied microbiology and Biotechnology*, 53(3): 278-281.

Pirt. S.J. 1975. *Principles of cell cultivation*. Blackwell Scientific London.

Prescott and Dunn. 1987. *Industrial microbiology*. 4th ed. CBS Publishers and Distributors, New Delhi, India. Pp. 550-565.

Rick, W. and H. P. Stegbauer. 1974. α – Amylase measurent of reducing groups. In H. V. Bergmeyer (ed.), Methods of enzymetic analysis, 2nd edn., Vol. 2, Academic press New York. Pp 885-890.

Snedecor, G.W. and W. G. Cochrane. 1980. Statistical methods. 7th edition. Ames, Iowa: Iwoa state University Press. ISBN 0-81381560-6.

Xuezhi, H., L. Chen, H. Qinfan and L. Zhoozu. 1991. Studies on the screening of thermostable alpha amylase producing *Bacillus strain. Acta-Microbiol. Sin, 31*(4): 267-273.

Yuguo, Z., L. U. C. Jianwei and L. I, Xiaoquin. 1993. Technology conditions of alpha amylase fermentation from hydrolyzate, *Shipin Yu Fajiang Gongue*, 6: 17-21.

Zhao, W. and J. Qirong. 1994. Selection and breeding of a high productivity strain of alpha-amylase from multiresistant mutant of *Bacillus. Wuxi Qinggongyo Xueguan Xuebao*, 13(1): 21-26.