



Strain Improvement Studies for the Production of L-Asparaginase by *Beauveria Bassiana* SS18/41

P. V. KamalaKumari*^{1,2}, G. Girija Sankar¹, T.Prabhakar¹

¹A. U. College of Pharmaceutical Sciences, Andhra University, Andhra Pradesh, India.

²Vignan Institute of Pharmaceutical Technology, Visakhapatnam, Andhra Pradesh, India.

*Corresponding author's E-mail: kamalaparavastu@gmail.com

Accepted on: 13-02-2015; Finalized on: 31-03-2015.

ABSTRACT

Strain improvement studies were conducted for the production of L-asparaginase from a marine fungus *Beauveria bassiana* SS18/14 by employing physical and chemical mutagens, in a systemic manner to obtain mutants that have higher L-asparaginase production. The wild strain produced 6.32 IU/mL of L-asparaginase activity while the UV mutant UVF-4 yielded 8.34 IU/mL and nitrous acid mutant UVF4-N-2 exhibited 10.44 IU/mL enzyme activity. The overall strain improvement programme increased L-asparaginase activity 1.65 times with respect to the parent wild strain.

Keywords: Physical mutagens, chemical mutagens, L-asparaginase, *Beauveria bassiana*

INTRODUCTION

Tumor cells, more specifically lymphatic cells, require huge amount of asparagine to maintain their rapid malignant growth. This means, they use both asparagine from the diet (blood serum) as well as what they can make themselves (which is limited) to satisfy their large L-asparagine demand. L-asparaginase as a drug exploits this unusually high requirement for the amino acid asparagine in tumor cells. L-asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia.^{1,2} as shown in Figure 1.

Healthy cells however escape unaffected, as they are capable of synthesizing asparagine themselves with the help of the enzyme L-asparagine synthetase, which is present in sufficient amounts.

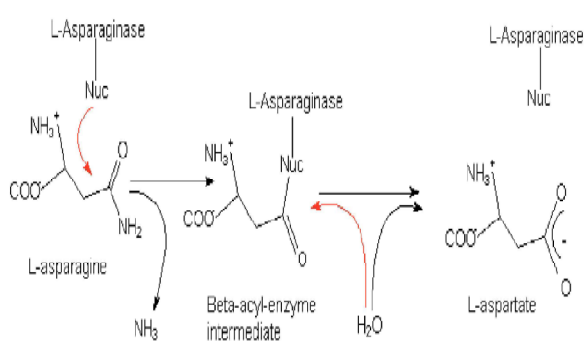


Figure 1: Mechanism of action of L-asparaginase

L-asparaginase arrests cell cycle in the G1 phase which was reported in the murine L5178Y cell line³ and the MOLT-4 human T-lymphoblastoid line⁴ resulting in apoptosis. A human acute lymphoblastic leukemia cell line is markedly inhibited by asparaginase, the effect being 10-fold higher for *Erwiniacaratovora* L-asparaginase.⁵ It has been reported that there is a requirement of a functional p53 protein for L-asparaginase to produce apoptosis as observed in studies

on HL60 pro myelocytic leukemia cell lines.^{6,7} However, another finding has strongly contradicted the general thought that the therapeutic benefit of L-asparaginase in leukemia is based on the fact that leukemic cells lack sufficient asparagine synthetase compared with normal cells.

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. Mutation is the primary source of all genetic variations and has been used extensively in industrial improvement for production. 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 microorganisms.

In the present study both physical (UV) and chemical (HNO₂) mutagens were employed in systemic manner to obtain mutants that gave higher L-asparaginase production. For UV irradiation, method⁸ was adopted. For chemical mutagenesis using HNO₂ method⁹ was followed. The parental strain SS18/41 was treated with mutagens UV and HNO₂ consecutively.

MATERIALS AND METHODS

UV irradiation of parent strain SS18/41 and selection of mutants

Strain improvement for the strain SS18/41 was done by mutation and selection. The strain was subjected to UV irradiation. The dose survival curve was plotted for selecting the mutants between 15% and 1% survivals. Mutation frequency was mentioned to be high when the survival rates were between 15 and 1%.

The spore suspension of wild strain was prepared in sterile distilled water and 4 mL quantities were pipetted aseptically in to sterile flat-bottomed Petri dishes of 100 mm diameter. The exposure to UV light was carried out in a Dispensing-Cabinet fitted with TUP 40 W Germicidal lamp that has about 90% of its radiation at 2540-2550 Å. 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, 5, 10, 15, 20, 25, 30 and 35 minutes respectively. During the exposure, the lid of the Petri dish was removed. Hands were covered with gloves and the plates were gently rotated so as to get uniform exposure of the contents of the Petri dish.

During the treatment, all the other sources of light were cut off and the exposure was carried out in dark. 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 distilled water, plated on to Sabouraud's Dextrose Agar (SDA) medium and incubated for 5 days at 28°C. The number of colonies in each plate was counted. It was assumed that each colony was formed from a single spore. Plates having survival rate between 15 and 1% were selected for the isolation of mutants.

The stable mutants were selected based on the consistent expression of the phenotypic character up to six generations and maintained on SDA medium for experimental purposes.

Nitrous Acid Treatment

The spore suspension of the parent strain was prepared by using acetate buffer pH 7.5. One mL of spore suspension was centrifuged, the supernatant was decanted and the spores were subjected to nitrous acid (0.1M sodium nitrite in phosphate buffer) treatment at intervals of 30, 60, 90, 120, 150 and 180 min, by incubating the mixture at 30°C.

After incubation, the suspension was centrifuged at 10,000 rpm, and the resultant pellet was washed twice with phosphate buffer (pH 7.0) and finally suspended in phosphate buffer. The samples were adequately diluted and plated on to SDA medium. The stable mutants were selected based on the consistent expression of the phenotypic character up to six generations and maintained on SDA slants. The plates were incubated at 28°C 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 SS18/41 strain was carried out by physical and chemical mutagenesis.

In the present investigation, the physical and chemical mutagens used were UV and nitrous acid consecutively.

Isolation of UV mutants and determination of L-asparaginase activity

The wild strain SS18/41 was subjected to UV irradiation at varying time intervals of 0, 5, 10, 15, 20, 25, 30, 35 min respectively. The number of survivals from each exposure time was represented in Figure 2. Plates having survival rate between 15 and 1% (35 min) were selected for the isolation of mutants as shown in Table 1. A total of 9 mutants were isolated and determined for their L-asparaginase production capacities by submerged fermentation and the activity was determined according to the method¹⁰. The results indicated that among the 9 UV mutants, UVF-4 showed highest L-asparaginase activity (8.34 IU/mL) when compared to parent wild strain (6.32 IU/mL) and the results were shown in Table 2 and Figure 3. Production of L-asparaginase by UVF4 mutant improved by 31.96% over the parent strain. Hence the best mutant strain UVF4 was selected for nitrous acid treatment for further improvement in L-asparaginase production.

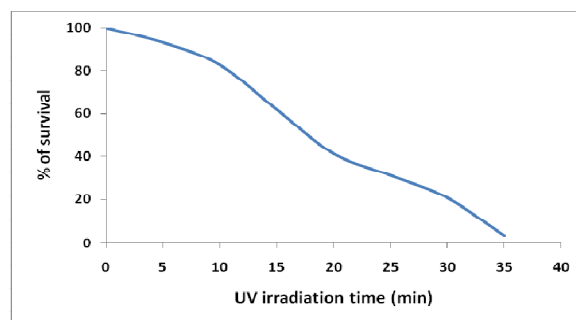


Figure 2: UV survival curve of the strain SS18/41

Table 1: Effect of UV irradiation on the strain SS18/41

Irradiation time (min)	Number of colonies/mL after irradiation ($\times 10^5$)	Percentage kill (%)	Survival percent (%)
0	29	0	100
5	27	6.9	93.10
10	24	17.34	82.66
15	18	38.01	61.99
20	12	58.68	41.32
25	9	69.01	30.99
30	6	79.34	20.66
35	1	96.56	3.44

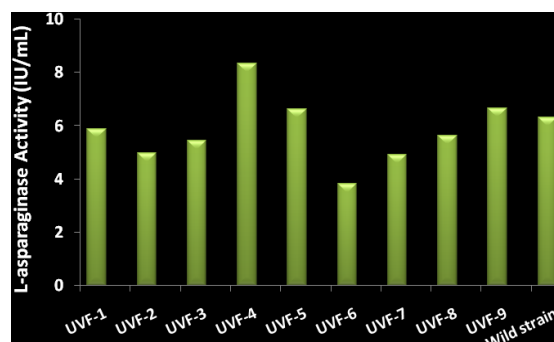


Figure 3: L-asparaginase activity of UV mutants from the strain SS18/41

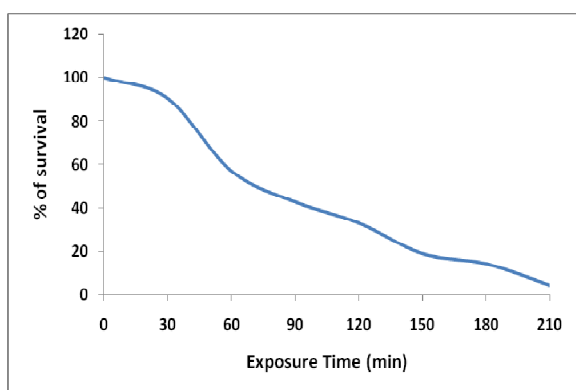
Table 2: L-asparaginase activity of UV mutants from the strain SS18/41

UV mutants	L-asparaginase activity (IU/mL)
UVF-1	5.88
UVF-2	4.98
UVF-3	5.46
UVF-4	8.34
UVF-5	6.62
UVF-6	3.84
UVF-7	4.92
UVF-8	5.64
UVF-9	6.66
Wild strain	6.32

Table 3: Effect of nitrous acid on UVF-4 from the strain SS18/41

Exposure time (min)	Number of colonies/mL after exposure	Percentage kill (%)	Percentage survival (%)
0	42	0	100
30	38	9.53	90.47
60	24	42.87	57.13
90	18	57.16	42.84
120	14	66.68	33.32
150	8	80.96	19.04
180	6	85.72	14.28
210	2	95.24	4.76

Isolation of nitrous acid mutants of UVF4 strain and determination of L-asparaginase activity

**Figure 4:** Survival curve of UVF-4 after nitrous acid treatment

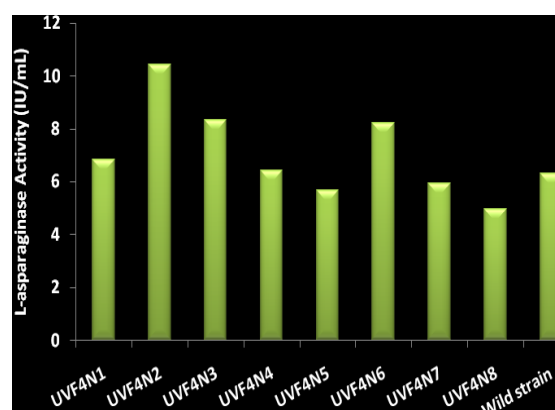
The selected mutant UVF4 was subjected to nitrous acid treatment. The number of survivals from each exposure time for UVF4 was represented in Figure 4. Plates having survival rate between 15 and 1% (180 and 210 min) were selected for the isolation of mutants as shown in Table 3. A total of 8 mutants were selected and determined for L-asparaginase activity. The results indicated that the mutant UVF4N2 yielded 10.44 IU/mL, which was 25.17%

improvement over the parent strain UVF4 (8.34 IU/mL) and the results were shown in Table 4 and Figure 5.

The wild strain produced 6.32 IU/mL of L-asparaginase activity while the UV mutant UVF-4 yielded 8.34 IU/mL and nitrous acid mutant UVF4-N-2 exhibited 10.44 IU/mL enzyme activity. The overall strain improvement programme increased L-asparaginase activity 1.65 times with respect to the parent wild strain.

Table 4: L-asparaginase activity of UVF-4 mutant from the strain SS18/41

UVF-4 mutant	L-asparaginase activity (IU/mL)
UVF4N1	6.84
UVF4N2	10.44
UVF4N3	8.34
UVF4N4	6.42
UVF4N5	5.68
UVF4N6	8.24
UVF4N7	5.92
UVF4N8	4.96
Wild strain	6.32

**Figure 5:** Production of L-asparaginase activity by various nitrous acid mutants of UVF

Isolation of nitrous acid mutants of UVF4 strain and determination of L-asparaginase activity

The selected mutant UVF4 was subjected to nitrous acid treatment. The number of survivals from each exposure time for UVF4 was represented in Figure 4. Plates having survival rate between 15 and 1% (180 and 210 min) were selected for the isolation of mutants as shown in Table 3. A total of 8 mutants were selected and determined for L-asparaginase activity. The results indicated that the mutant UVF4N2 yielded 10.44 IU/mL, which was 25.17% improvement over the parent strain UVF4 (8.34 IU/mL) and the results were shown in Table 4 and Figure 5.

The wild strain produced 6.32 IU/mL of L-asparaginase activity while the UV mutant UVF-4 yielded 8.34 IU/mL and nitrous acid mutant UVF4-N-2 exhibited 10.44 IU/mL enzyme activity. The overall strain improvement

programme increased L-asparaginase activity 1.65 times with respect to the parent wild strain.

CONCLUSION

High yielding strains were developed through mutagenesis by employing physical (UV rays) and chemical mutagens (Nitrous acid). The wild strain SS18/41 produced 6.32 IU/mL of L-asparaginase activity while the UV mutant UVF-4 yielded 8.34 IU/mL and nitrous acid mutant UVF4-N-2 exhibited 10.44 IU/mL enzyme activity. The overall strain improvement programme using UV irradiation and HNO₂ treatment increased L-asparaginase activity 1.65 times with respect to the parent wild strain SS18/41.

REFERENCES

1. Kiriya Y, Kubota M, Takimoto T, Kitoh T, Tanizawa A, Akiyama Y, Mikawa H, Biochemical characterization of U937 cells resistant to L-asparaginase: the role of asparagine synthetase, *Leukemia*, 3(4), 1989, 294-297.
2. Campbell H A, Mashburn, E A, Boyse, and Old LJ, Two L-asparaginases from *Escherichia coli* B. their separation, purification, and antitumor activity, *Biochemistry*, 6, 1967, 721-730.
3. Ueno T, Ohtawa K, Mitsui K, Kodera Y, Hiroto M, Matsushima A, Inada Y, Nishimura H, Cell cycle arrest and apoptosis of leukaemia cells induced by L-asparaginase, *Leukemia*, 11, 1997, 1858-1861.
4. Shimizu T, Kubota M, Adachi S, Sano H, Kasai Y, Hashimoto H, Akiyama Y, Mikawa H, Pre-treatment of a human T-lymphoblastoid cell line with L-asparaginase reduces etoposide-induced DNA strand breakage and cytotoxicity, *International Journal of Cancer*, 50, 1992, 644-648.
5. Ohnuma T, Bergel F, Bray RC, Enzymes in cancer, Asparaginase from chicken liver, *Biochem J.* 103(1), 1967, 238-245.
6. Fu C, Nandy P, Danenberg P, Avramis, V, Asparaginase (ASNase) induced cytotoxicity depends on the p53 status of the cells, *Proceedings of the American Association for Cancer Research*, 39, 1998, 602.
7. Nandy P, Fu C, Danenberg P, Avramis VI, Apoptosis induced by antimetabolites, taxanes or asparaginases *in vitro* depends on the p53 status of the leukaemic cells, *Proceedings of the American Association for Cancer Research*, 39, 1998, 602.
8. Parekh S, Vinci VA, Strobel RJ, Improvement of microbial strains and fermentation process, *Applied Microbiology and Biotechnology*, 54, 2000, 287.
9. Sukumaran CP, Singh DV, Mahadevan PR, Synthesis of L-asparaginase by *Serratia marcescens* (Nima), *J. Biosci.* 1(3), 1979, 263-269.
10. Imada A, Igarasi S, Nakahama K and Isono M, Asparaginase and glutaminase activities of micro-organisms, *J Gene Microbiol*, 76, 1973, 85-99.

Source of Support: Nil, Conflict of Interest: None.

