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# STRAIN IMPROVEMENT OF ISOLATED *BACILLUS SUBTILIS STRAIN HSWX88* FOR EXTRACELLULAR L-ASPARAGINASE PRODUCTION

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## Abstract

The purpose of the present investigation is to enhance extracellular L-asparaginase production by subjecting *Bacillus subtilis strain hswx88,* isolated from Taptapani, a hot spring of Ganjam District of Odisha, India, to improvement by random mutagenesis by ultra-violet (UV) irradiation, Nitrous acid and N-Methyl-N'-nitro-N-nitroso guanidine (NTG) treatment. Mutants were screened as L-asparaginase producers on the basis of enzyme activity in submerged fermentation. UVS-5 mutant obtained after 18-20 minutes of UV exposure showed 1.25 times more enzyme activity (81.46 IU/ml). UVS-5 mutant further mutated by Nitrous acid and UVS5-N-6 mutant obtained after 210-240 Sec Nitrous acid exposure showed 1.59 times increase in enzyme activity (104. 06 IU/ml). Then UVS5-N-6 mutant was treated with NTG. *UVS5-N-6-N-9* mutant obtained after 210-240 minute NTG exposure showed 2.03 times increase in enzyme activity (132.62 IU/ml) in compared to the isolated wild *Bacillus subtilis strain hswx88* (65. 28 IU/ml). The results indicated that UV radiation, Nitrous acid and NTG were effective mutagenic agents for improvement of *Bacillus subtilis strain hswx88*. Thus these findings have more impact on enzyme economy by enhancing the production of extracellular L-asparaginase for anticancer applications.

**Keywords:** *Bacillus subtilis strain hswx88,* Mutation, L-asparaginase, Strain improvement & Leukemia.

## Introduction

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) is an important component in the treatment of certain kinds of cancer therapies like acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma chemotherapy, pancreatic carcinoma, melanosarcoma, reteclesarcoma (Athale, *et.al*. ,2003, Narta *et.al*., 2007 & Chakrabarti, 1997). The antineoplastic activity of l-asparaginase is associated with the property of depleting the circulating pool of L-asparagine to aspartic acid and ammonia by its catalytic activity (Aslanian *et.al*., 2001). Malignant

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cells with low L-asparagine levels are killed due to lack of an exogenous supply of this amino acid combined with an impaired protein synthesis mechanism. However, normal cells are protected from L-asparagine-starvation due to their ability to produce this amino acid. Based on this, L-asparaginase has also been included in most contemporary, multi-agent regimens for adult acute lymphoblastic leukemia (ALL). L-asparaginase is also used as an L-asparagine biosensor for leukaemia cells (Verma *et.al.,* 2007), and for the production of acrylamide-free food in the food industry (Pedreschi *et.al.*, 2008). Microbial L-asparaginase has received the greater attention because of its apparent advantages in production and purification at large scale in addition to its antitumor property (Kavitha *et.al.,* 2012). L-asparaginase has attracted much attention for bulk production with low cost, due to its wide range of applications.

Mutation is the primary source of all genetic variation and has been used extensively in industrial improvement of enzyme production (Lee *et.al*., 2007). 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 to applied genetics involved trial and error. The major motivation for industrial strain development is economic, since the metabolic concentrations produced by wild strains are usually too low for economical process (Lin *et.al.*, 2011). Wild strains frequently produce a mixture of chemically closed related substances. Mutants who synthesize one component as the main product are preferable, since they make possible a simplified process for product recovery (Khushoo *et.al.,* 2004). Changes in the genotype of microorganisms can lead to the biosynthesis of new metabolites with increased yield (Zambare *et.al.,* 2012). Genetic variations can be produced by mutation and genetic recombination. The success of strain development depends on an optimal use of mutagenesis (production of mutations) procedures in combination with an effective system for selecting high yielding strain. There were scanty reports on mutational studies for enhancement of L-asparaginase production. Therefore, the present investigation demonstrates the effectiveness of ultra-violet (UV) irradiation (Hungund *et.al.,* 2010), Nitrous acid and N-Methyl-N'-nitro-N-nitroso guanidine (NTG) (Krishna *et.al.,* 2011) in strain improvement for enhanced production of L-asparaginase by *Bacillus subtilis strain hswx88*. The mutants were compared with wild type for L-asparaginase production.

## Materials and Methods

### *Microorganisms*

The thermophilic bacteria *Bacillus subtilis strain hswx88* (Gene Bank Accession Number: JN412064.1) isolated from Taptapani hotspring of Odisha, India was employed in the present study. The strain was grown on nutrient agar medium slants at 37°C for two days and used for the study. The slants were sub cultured at monthly intervals and stored at $4^0$C in the refrigerator.

### *Cultivation medium and cultural conditions*

The composition and of initial cultivation medium was (g/l): L-asparagine - 8, lactose - 3.6, peptone - 1.09, gelatin - 1.07, $Na_2HPO_4.2H_2O$ - 6.0, $KH_2PO_4$ - 3.0, NaCl - 0.5, $MgSO_4.7H_2O$ - 0.5, $CaCl_2.2H_2O$ - 0.015and initial pH was maintained at 7.0 (Pradhan *et.al.*, 2013, 'a') with incubation temperature 40°C, inoculums age of 16h, inoculums size of 6 % w/v, an agitation speed of 150 rpm and 80% air space. The inoculum was prepared by adding loopful of selected isolates into 50ml sterilized medium specified above in 250 ml Erlenmeyer (EM) flasks. The flasks were agitated at 150 rpm and incubated at 37°C for 12 h ($O.D._{600}$ nm=0.6-0.8) in an orbital shaker incubator (Pradhan *et.al.,* 2013, 'a'). After incubation the cells were removed by centrifugation at 6000 × g for 10 min at 4°C. Then the supernatant collected was subjected to assay of extracellular L-asparaginase production.

### *Enzymatic Assay*

L-asparaginase activity was measured by direct Nesslerization of ammonia. The activity of L-asparaginase was measured employing the modified method of Wriston (Pradhan *et.al.,* 2013, 'b').

### Preparation of Cell Suspension

The organism grown on nutrient agar slants were scraped off into sterile water containing tween 80 (1:4000) to give a uniform suspension. The suspension was transferred into a sterile conical

flask and thoroughly shaken for 30 min on a rotary shaker. This cell suspension was used for mutation studies.

**Mutation and Selection**

**Mutation by UV irradiation**

The cell suspension of wild strain was prepared in 5 mL phosphate buffer; (pH 5.5) from such slant. The suspensions were pooled together from a number of slant and four mL quantities were pippetted aseptically into sterile petridishes of 100 mm diameter, kept over a flat surface. The exposure to UV light was carried out in a "Dispensing Cabinet" fitted with UV lamp TUP 40W germicidal lamp which has about 90% of its radiation at 2540-2550 $A^0$. The exposure was carried out at a distance of 26.5cm away from the center of the Germicidal lamp. The exposure times were 0, 3, 6, 9, 12, 15 and 18 min respectively. During the exposure, the lid of the Petridish was removed. Hands were covered with gloves and the plates were gently rotated so as to get uniform exposure to the UV rays. During the exposure all the other sources of light were cut off and the exposure was carried out in dark under red light. The treated cell suspension was transferred into sterile test tubes covered with a black paper and kept in the refrigerator overnight to avoid photo-reactivation.

Each irradiated cell suspension was serially diluted with sterile phosphate buffer. The cell suspension after diluting in a suitable buffer was plated onto nutrient agar medium and incubated for 24 h at $37^0$. The number of colonies in each plate was counted. The % of survivals from each exposure time is shown in Fig. 1. A % survival curve was plotted. Plates having less than 1% survival rate (after 15-18 min exposure) were selected for the isolation of mutants. About 12 isolates (UVS1-UVS12) were selected on the basis of macroscopic differences transferred onto nutrient agar slants and tested for their production capacities as described earlier. The best L-asparaginase producing UV mutant strain was selected for further nitrous acid treatment.

**Nitrous acid treatment**

The cell suspension of the selected strain UVS5 was prepared by using Acetate buffer pH-7. To 9ml of the cell suspension in buffer 1ml sterile stock solution of 0.01 M Sodium Nitrite was added. Sample of 4ml were withdrawn at 30, 60, 90, 120, 150, 180, 210 and 240 second respectively. Each 1ml sample was neutralized with 0.5ml of 0.1M NaOH, serially diluted and plated on the nutrient agar medium.

The colonies from each sample were counted and a % survival curve was plotted and shown in Fig. 2. Plates having survival rate between 15 and 1% were selected for the isolation of mutants. The stable mutants UVS5-N-1 to UVS5-N-10 were selected based on the consistent expression of the phenotypic character up to six generations and maintained on nutrient agar slant. The plates were incubated at 37°C for 24 hours.

**NTG treatment and selection of mutants**

The NTG is considered to be a very effective mutagen (Wang *et al.*, 2007), it induces linked multiple mutations at fairly high frequencies and promotes base pair substitutions, primarily GC-AT transitions in microorganisms. The time of exposure was 2 to 3 h and the temperature of incubation was $37^0$C.

The Nitrous acid mutants of UVS5-N-6 maintained on nutrient agar slants was used for NTG treatment. The selected Nitrous acid treated mutant (UVS5-N-6) was sub-cultured onto nutrient agar and incubated at $37^0$C for 24 h. The cell suspension was prepared as described earlier. The cells were suspended in sterile phosphate buffer and shaken to get a uniform suspension.

The NTG (100 mg) was accurately weighed and dissolved in 25 mL of phosphate buffer at a temperature of $40^0$C to minimize decomposition and sterilized by passing through sterile bacterial filter (0.22 µm). Ten mL of the cell suspension was added to 25 mL of NTG solution and immediately incubated at $37^0$C in a water bath. At appropriate time intervals 5 mL samples were withdrawn and centrifuged immediately. The supernatant was decanted, and then the cell pellet was washed with sterile distilled water and finally resuspended in 5 mL of sterile distilled water. A total of seven samples were withdrawn from the cell suspension each in 5 mL quantities after exposure to NTG for 30, 60, 90, 120, 150, 180, 210 and 240 min respectively. A control also was included without exposure to NTG.

The above treated samples were serially diluted and 100 µL of each sample was spread onto NA plates by spread plate technique. The plates were incubated at 37$^0$C for 24 h. The colonies from each plate were counted and a % survival curve was plotted and shown in Fig. 3. Plates having less than 1% survival rate were selected for the isolation of mutants. Colonies were selected on the basis of macroscopic characteristics. A total of 12 colonies were selected, picked up from the plates and transferred onto nutrient agar slants. These were labeled as *UVS5-N-6-N-1* to *UVS5-N-6-N-12*. They were incubated at 37$^0$C for 24 h to get good growth. All the selected mutants were tested separately for their L-asparaginase production capacity.

**Statistical analysis**

All values presented here are the average values of triplicate analysis.

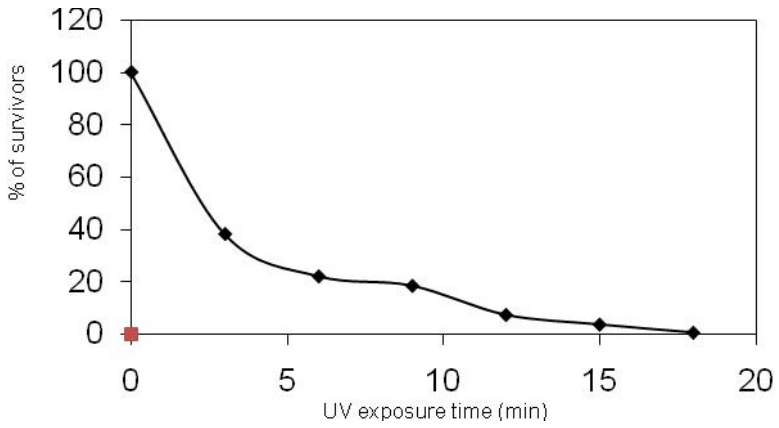


**Fig. No. 01: Effect of UV irradiation on *Bacillus subtilis strain hswx88***

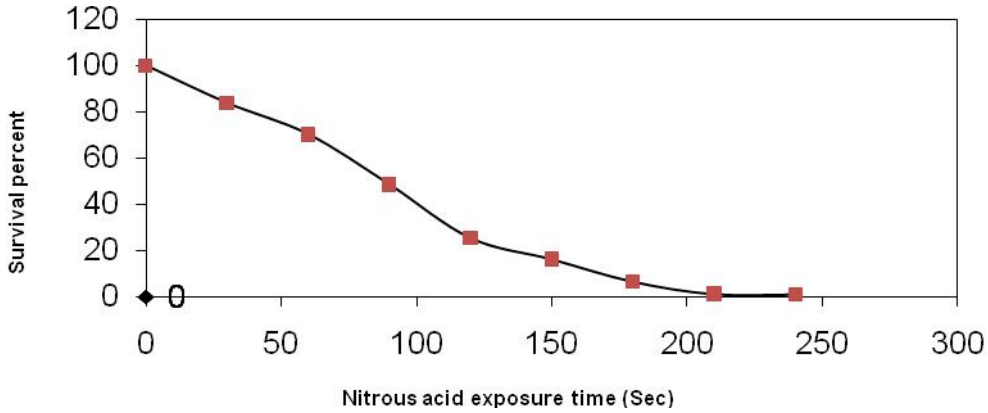


**Fig. No. 02: Effect of Nitrous acid on UVS5 *Bacillus subtilis strain hswx88***

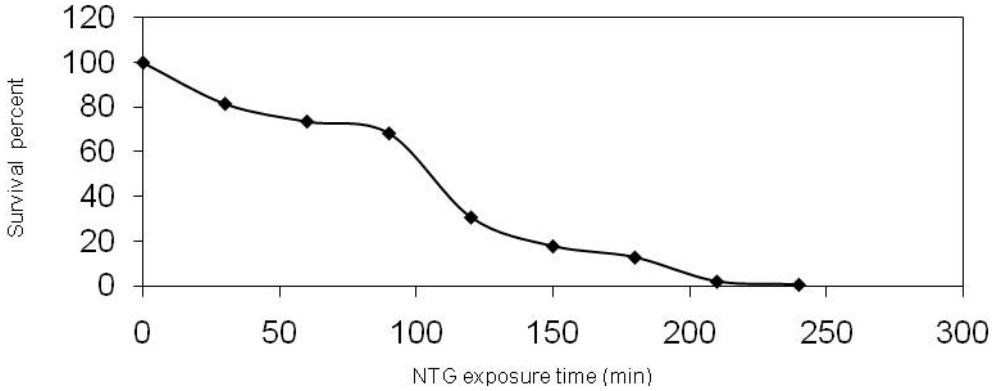


**Fig. No. 03: Effect of NTG on UVS7-N-6 *Bacillus subtilis strain hswx88***

**Table No. 01: L-asparaginase production capacity of UV mutants**

| UV mutants | L-asparaginase activity (IU/mL) |
|---|---|
| UVS1 | 69.24 |
| UVS2 | 62.44 |
| UVS3 | 60.06 |
| UVS4 | 73.48 |
| **UVS5** | **81.46** |
| UVS6 | 76.72 |
| UVS7 | 68.5 |
| UVS8 | 62.78 |
| UVS9 | 76.09 |
| UVS10 | 70.46 |
| UVS11 | 63.38 |
| UVS12 | 72.86 |
| *Bacillus subtilis strain hswx88* | 65.28 |

**Table No. 02: L-asparaginase production capacity of Nitrous acid mutants**

| Nitrous acid Mutants | L-asparaginase activity (IU/mL) |
|---|---|
| *UVS5-N-1* | 86.34 |
| *UVS5-N-2* | 90.02 |
| *UVS5-N-3* | 92.48 |
| *UVS5-N-4* | 88.42 |
| *UVS5-N-5* | 96.06 |
| ***UVS5-N-6*** | **104.06** |
| *UVS5-N-7* | 80.76 |
| *UVS5-N-8* | 78.05 |
| *UVS5-N-9* | 88.68 |
| *UVS5-N-10* | 74. 88 |
| *Bacillus subtilis strain hswx88* | 65.28 |

**Table No. 03: L-asparaginase production capacity of NTG mutants**

| NTG Mutants | L-asparaginase activity (IU/mL) |
|---|---|
| *UVS5-N-6-N-1* | 120.86 |
| *UVS5-N-6-N-2* | 112.64 |
| *UVS5-N-6-N-3* | 98.26 |
| *UVS5-N-6-N-4* | 88.44 |
| *UVS5-N-6-N-5* | 115.09 |
| *UVS5-N-6-N-6* | 88.46 |
| *UVS5-N-6-N-7* | 96.36 |
| *UVS5-N-6-N-8* | 88.96 |
| ***UVS5-N-6-N-9*** | **132.62** |
| *UVS5-N-6-N-10* | 108.67 |
| *UVS5-N-6-N-11* | 78.34 |
| *UVS5-N-6-N-12* | 72.68 |
| *Bacillus subtilis strain hswx88* | 65.28 |

## Results and discussion

The wild strain of *Bacillus subtilis strain hswx88* was subjected to strain improvement programme with a view to obtain increased L-asparaginase production and to achieve greater stability of the organism. The first method chosen was UV irradiation.

### Isolation of UV Mutants and their L-asparaginase Activity

The selected mutants were provided with the code number *UVS1* to *UVS12* and these mutants were tested for their L-asparaginase producing capabilities and Results are prescribed in Table 1. The results indicated that among UV-mutants, *UVS5* was found to be the highest L-asparaginase producer (81.46 IU/mL) and it was 1.25 times

higher than the parent strain (65.28 IU/mL). The mutant, *UVS5* was selected for further strain improvement studies using Nitrous acid.

**Selection of Nitrous acid Mutants and their L-asparaginase Activity**

The selected mutant *UVS5* was subjected to nitrous acid treatment. Mutation frequency was observed to be high when the survival rates were between 15 and 1%. The dose survival curve was plotted for selecting the mutants between 15% and 1% survivals. Plates having survival rate between 15 and 1% (210 and 240 sec) were selected for the isolation of mutants. The survival curve was plotted by taking Exposure time (Second) on X-axis and % survival on Y-axis. (Fig 2.)

A total of 10 mutants were selected and determined for their L-asparaginase production capacities by submersed fermentation and presented in Table 2. Out of 10 mutants *UVS5-N-6* showed maximum L-asparaginase activity of 104.06 IU/mL, which was 1.59 times more than the wild isolate *Bacillus subtilis strain hswx88*(65.28 IU/mL).

**Selection of NTG Mutants and their L-asparaginase Activity**

The selected UV & Nitrous acid-mutant, *UVS5-N-6* was subjected to NTG treatment. A total of 12 mutants were selected and they were provided with the code numbers *UVS5-N-6-N-1* to *UVS5-N-6-N-12*. These were evaluated for their L-asparaginase production capacities. The results are shown in Table 3. The results indicated that the mutant *UVS5-N-6-N-9* was the highest L-asparaginase yielding strain (132.62 IU/mL). Thus the NTG treatment resulted a mutant that produced 2.03 times higher L-asparaginase than the parent wild strain (65.28 IU/mL), which is a significant increase in yield. From the results, it is evident that UV, Nitrous acid and NTG were effective mutagenic agents for strain improvement of *Bacillus subtilis strain hswx88*. By employing these techniques, a superior mutant (*UVS5-N-6-N-9*) with a productivity of 2.03 times higher yield than the parent strain was obtained.

**Summary**

The major motivation for industrial strain development is economic, since the metabolic concentrations produced by wild strains are usually too low for economical process. Wild strains frequently produce a mixture of chemically closed related substances. Mutants who synthesize one component as the main product are preferable, since they make possible a simplified process for product recovery. Changes in the genotype of microorganisms can lead to the biosynthesis of new metabolites with increased yield. Genetic variations can be produced by mutation and genetic recombination. The success of strain development depends on an optimal use of mutagenesis (production of mutations) procedures in combination with an effective system for selecting high yielding strain.

L-asparaginase is an enzyme used as an antitumor agent in the treatment of various types of leukemia. Earlier, this enzyme was produced by several bacteria but for commercial production only mutants of *E. coli* were used. The shortage of this enzyme, subsequently lead to the discovery of novel organisms producing L-asparaginase. Here we have used thermophilic isolate *Bacillus subtilis strain hswx88*, obtained from Taptapani hot spring of Odisha, India for the enhanced production of L-asparaginase. There are many evidences on the successful employment of strain improvement techniques to increase product yield in various industrially important microorganisms (Mocktar *et.al.,* 2007). We employed Physical (UV radiation) and chemical (Nitrous acid & N-methyl-N-nitro-N-nitrosoguanidine) mutagens to induce mutations in *Bacillus subtilis strain hswx88*, which is a very good producer of extracellular L-asparaginase. The effectiveness of the mutagens helped us to achieve a **2.03** times enhanced production of L-asparaginase in the mutated strain than the wild strain.

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