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STRAIN IMPROVEMENT OF ALKALINE PROTEASE FROM *TRICHODERMA REESEI* MTCC-3929 BY PHYSICAL AND CHEMICAL MUTAGEN

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ABSTRACT

The purpose of the present investigation is to enhance alkaline protease production by subjecting indigenous protease producing strain Trichoderma reesei MTCC-3929 to improvement by random mutagenesis by ultra-violet (UV) irradiation and N-Methyl-N'-nitro-N-nitroso guanidine (NTG) treatment. Mutants were screened as protease producers on the basis of zone of clearance on skimmed milk agar plates. UV-8 mutant showed 9 mm clear zone diameter and activities of 199.6 and 552.6 U/ml for submerged fermentation (Smf) and solid state fermentation (SSF), respectively. UV-8 further mutated by NTG to produced NTG-17 mutant with zone of clearance 13mm diameter. Compared to wild strain, NTG-17 mutant was found to produce 2.6 and 2.2-fold more activities in SmF and SSF, respectively. Thus these findings have more impact on enzyme economy for biotechnological applications of microbial proteases.

Keywords: *Trichoderma reesei; strain improvement; UV-NTG mutation; alkaline protease*

[I] INTRODUCTION

Proteases, which account for about 60% of total enzyme market and among the most valuable commercial enzymes, are the single largest class of enzymes occupying a pivotal position due their wide application in the industrial processes [1]. Plants, animals and microbial sources are employed for protease production. Microbes serve as the preferred source of proteases because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties. Microbial extracellular proteases are important enzymes and are mainly used in detergents to facilitate the release of proteinaceous stains such as blood, milk, egg and meat. Today, proteases account for approximately 40% of the total enzyme sales in various industrial market sectors, such as detergent, food, pharmaceutical, leather, diagnostics, waste management and silver recovery. This dominance of proteases in the industrial market is expected to increase further by the year 2005 [2]. However, until today, the largest share of the enzyme market has been held by detergent alkaline proteases active and stable in the alkaline pH range [3]. As only few reports are available on the use of fungal proteases in detergent industry, therefore there is a growing need to exploit fungal proteases for commercial exploitation in detergent industry.

For industrial use enzyme must be produced at low cost and should be reusable and reproducible. To achieve this many techniques have been developed for protease improvements. Strain improvement is usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, γ-rays, UV rays, *etc.*, and chemical mutagens such as NTG, EMS, etc. [4].

The mutation of fungal strain for various industrial enzymes (lipase, chitinase, cellulase, glucoamylase etc) has been widely used in many investigations [5-8]. But there was no report available on mutation studies of *Trichoderma reesei* for protease productions. Also very less work was published on protease improvement from fungi using classical mutation.

In the present study, the alkaline protease production by *T. reesei* has been improved by physical and chemical mutagen

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to exploit its use in detergent industry. The effect of mutation on fungi was evaluated in Smf and SSF.

[II] MATERIALS AND METHODS

2.1. Microorganism and growth media

Trichoderma reesei MTCC-3929 was procured from Microbial Type Culture Collection (MTCC), Chandigarh (India). It was grown on Potato Dextrose agar (PDA) at 30°C, and then stored at 4°C. PDA slants, incubated for 7 days, were used for the preparation of the inoculum.

2.2. Preparation of spore suspension

Ten milliliters of 0.85% saline containing 0.1% Tween-80 was transferred to a sporulated (7 day old) PDA slant culture of *T. reesei*. The spores were dislodged using an inoculation needle, under aseptic conditions, and the suspension was used for its spore count by serial dilution and plating on PDA agar medium.

2.3. Protease screening

Protease production by *T. reesei* (wild and mutants) were tested on skimmed milk agar plates containing (g/L) 4 potato infusion, 20 dextrose, 10 skimmed milk, 20 agar (pH 5.6).

2.4. Enzyme assay

The protease activity was determined by caseinolytic assay method of [9]. The cell free supernatant (1ml) was mixed with 4ml of casein (0.625% w/v) and incubated at 40°C for 30min. The reaction was stopped by addition of 5ml of 5% trichloroacetic acid. Enzymatically hydrolyzed casein was measured by modified Folin Ciocalteu method [10], against casein treated with inactive enzyme as blank. A standard graph was generated using standard tyrosine solutions of 5–50 µg ml^{-1}. One unit of protease activity was defined as the amount of enzyme which liberated 1 µg tyrosine per min at 40°C.

2.5. Isolation and selection of mutants

2.5.1. UV irradiation

Four ml of the spore suspension containing 10^7spores/ml was pipetted aseptically into sterile petri-dish of 80 mm diameter having a flat bottom. The exposure of spore suspension to UV light was carried at distance of 30 cm away from UV lamp (15W, 2537A^0). The exposure times were 5, 10, 15, 20 and 25 min. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation, then was serially diluted in saline and plated on PDA medium. The plates were incubated for 7 days at 30°C and the numbers of colonies in each plate were counted. Each colony was assumed to be formed from a single spore. Mutants were selected from the plates showing less than 1% survival rate and screened for protease production on skimmed milk agar plates.

2.5.2. NTG treatment

The best UV mutant (UV-8) was used for NTG treatment. The spore suspension was prepared in the same manner as described earlier and calculated the spore count. To 9 ml of spore suspension (10^6 spores/ml), 1 ml NTG solution (10 mg/ml in sterile water) was added. The reaction was allowed to proceed at 30^{0}C, 120 rpm. Samples were withdrawn from the reaction mixture at intervals of 20, 40, 60, 80 and 100 min and immediately centrifuged for 10 min at 5000 rpm and the supernatant solution was decanted. Cells were washed three times with sterile water and resuspended in 10 ml of sterile saline. The samples were serially diluted in the same saline and plated over PDA as mentioned earlier. NTG mutants were selected from the plates showing less than 1% survival rate and screened for protease production on skimmed milk agar plates.

2.6. Protease production

Hyper producing mutant of UV, NTG treatment and a wild strains were inoculated separately in Smf and SSF medium. The liquid medium was prepared which containing (g/l) 5 wheat bran, 10 soybean meal, 0.1 yeast extract, 2 KH_2PO_4, 4 K_2HPO_4, 0.5 NaCl, 0.1 $MgSO_4$, 2 $CaCl_2$ (pH 7) and the solid medium contained 10 g wheat bran with 10 ml water. Both fermentations were performed at 30^{0}C up to 5 days under shaking and static conditions for Smf and SSF, respectively. For solid medium, the fermented koji was mixed with 100 ml water and kept for 2h at room temperature. After 2h, it was filter off through cotton filter. The filtrate was used for protease activity measurements.

2.7. Statistical analysis

All values presented here are the average values of triplicate analysis ± standard deviation (SD).

[III] RESULTS AND DISCUSSION

3.1. UV Mutation

The UV treatment with fungal spores showed 1% survival when treated for 20 min **[Figure-1]**. The plates having less than 1% survival rate was observed at 20 min UV exposure time. Hopwood et al., suggested that 99.9% kill is best suited for strain improvement as the fewer survivors in the treated sample will have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the culture [11].

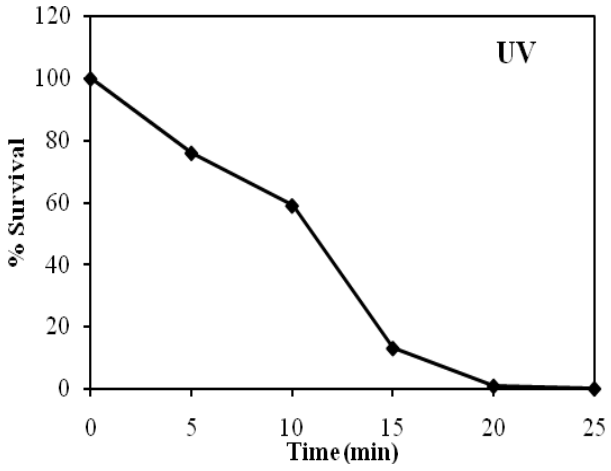

Fig: 1. UV irradiation on survival of *T. reesei* at different time (30 cm distance, 4 ml spore suspension)

From UV mutagenesis total 20 mutants were selected on the basis of different morphology, shapes and colored mutants.

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Out of 20 mutants, only two mutants (UV-3 and UV-8) showed higher clear zone diameter than wild type **[Table-1]**. UV-8 showed maximum protease activities of 199.6±6.5U/ml (in Smf) and 552.6±3.5U/ml (in SSF), respectively. The wild strain produced protease activities of 121.3±4.1U/ml (in Smf) and 344.3±5.6U/ml (in SSF), respectively. Overall, 1.6-fold protease activity was enhanced from UV-8 mutant in Smf as well as SSF, when compared with wild strain. Djamel et al. reported hyper-producing acid protease UV mutant (S08M4) that produced 1400U/ml in liquid fermentation [12]. UV mutation was also used for production of alkaline protease from *Bacillus* sp. [13].

Table 1. UV mutant and this protease activity

Isolates	Zone of clearance (MM)	Protease activity (U/ml)	
		[b]SmF	[c]SSF
Wild strain	5	121.3±4.1[e]	344.3±5.6
[a]UV-1	4	102.3±5.8	245.6±4.5
UV-2	3	77±5.1	154.3±5
UV-3	6	126.3±8.7	349±2.6
UV-4	1	35.6±6.1	84.6±3.7
UV-5	5	111±4.3	203.6±2.5
UV-6	5	83.69.2	187.3±4
UV-7	[d]+	10±2.6	41.6±4
UV-8	9	199.6±6.5	552.6±3.5
UV-9	+	0	8.3±2
UV-10	+	0	20±3.6
UV-11	4	84±5.5	237.6±3.5
UV-12	3	71.6±6.6	179±6.5
UV-13	3	66.6±5.5	195.3±4.9
UV-14	1	19±4.5	81.6±4
UV-15	2	41.6±3.5	142±3.6
UV-16	5	98±6	302.6±3.7
UV-17	1	7.6±1.5	36.6±2.3
UV-18	2	20.6±2.3	70.6±2.5
UV-19	3	40.3±2.5	159.3±3.5
UV-20	+	0	14.3±2.5

[a]UV-Ultra-violet, [b]Smf-submerged fermentation, [c]SSF-solid state fermentation, [d]protease positive, [e]Mean ± SD

3.2. NTG mutation

The UV mutant (UV-8) was selected and was subjected for further strain improvement by NTG treatment. NTG was considered to be very effective chemical mutagen. The 1% survival was observed in between 60-80 min incubation **[Figure-2]**.

Total 20 mutants were selected on SMA plates. Among 20 mutants, one mutant (NTG-17) showed maximum clear zone (13 mm diameter) than UV-8 mutant while 8 mutants showed higher clear zone diameter than wild strain. NTG-17 showed maximum protease activities of 318.3±2.5U/ml (in Smf) and 747.3±3.2U/ml (in SSF), respectively. The protease yield of NTG-17 mutant was 1.6 and 1.4-fold higher in Smf and SSF than UV-8 mutant **[Table-2]**. When compared to wild strain, NTG mutant showed 2.6 and 2.2-fold higher protease activities in Smf and SSF. Likewise, but higher fold (3.5) protease activities were reported by UV/NTG mutant of *Bacillus pumilus* [14]. Ryden et al. reported an increase in protease production of 3-10 folds by using a *Staphylococcus aureus* mutant generated by NTG [15].

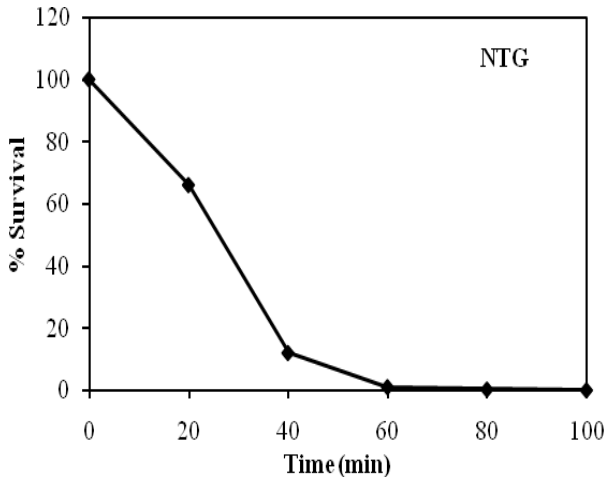

Fig: 2. NTG treatment on survival of UV mutant of *T. reesei* (UV-8) at different time (NTG, 3 mg/ml, 10 ml spore suspension)

Table 2. NTG mutant and this protease activity

Isolates	Zone of clearance MM)	Protease activity (U/ml)	
		[c]SmF	[d]SSF
Wild strain	5	124.6±4.1[e]	348.6±7
[a]UV-8	9	196.3±8.5	549.3±3.5
[b]NTG-1	4	116.3±2.5	324.3±7.5
NTG-2	5	125.3±4	355.6±0.5
NTG-3	6	145.3±3.2	402.3±3.2
NTG-4	5	124.3±4.7	350.3±1.5
NTG-5	9	190.6±2.8	482.6±5
NTG-6	3	83.6±4.5	201.6±2
NTG-7	1	37.6±4.1	97.3±3.7
NTG-8	2	46±2	145.3±3.2
NTG-9	6	151.6±3	246.6±3
NTG-10	4	112±0.5	135±3
NTG-11	3	84.3±3.5	83.3±5.1
NTG-12	4	121.6±1.5	145.6±1.5
NTG-13	2	36±3	121±2
NTG-14	6	143.6±2.5	254±5
NTG-15	4	43±2	341±3
NTG-16	7	180.6±1.5	336.3±3
NTG-17	13	318.3±2.5	747.3±3.2
NTG-18	2	42.6±1.5	144.3±1.5
NTG-19	5	121.3±3.2	336.3±2.5
NTG-20	6	146.6±4.9	384.3±4.5

[a]UV-Ultra-violet, [b]NTG- N-Methyl-N'-nitro-N-nitroso guanidine, [c]Smf-Submerged fermentation, [d]SSF-solid state fermentation, [e]Mean ± SD

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To the best of our knowledge there was no report available on strain improvement of *T. reesei* for protease production. Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the processes with increased productivity and may also possess some specialized desirable characteristics. Effectiveness of UV Irradiation (physical mutagen) and NTG treatments (Chemical mutagen) in strain improvement for enhanced protease productivity was demonstrated in the present investigation. It is hoped that the high yielding fungal mutant strain of the isolate *T. reesei* (NTG-17) can be exploited commercially for large-scale industrial production of protease.

[IV] CONCLUSIONS

A mutant, NTG-17 was obtained from a Mutant UV-8 of *T. reesei* ATCC-3929. It was screened as best potential because of its enhanced zone of clearance on skimmed milk agar plate. It was developed using UV irradiation followed by NTG treatment of the spores Compared to wild strain, NTG-17 mutant showed 2.6 and 2.2-fold higher protease production in Smf and SSF, respectively. Thus the selected mutant has potential in minimizing the cost of enzyme for its biotechnological applications.

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