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Development of mutant fungal strains of *Aspergillus niger* for enhanced production of acid protease in submerged and solid state fermentation

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ABSTRACT

The acid protease productivity was enhanced by altering the genetic nature of wild type fungal strain A. niger by conventional mutation methods. Two methods viz. chemical and physical methods were employed to develop mutant strains on casein – agar medium. About eleven chemical mutant fungal strains were obtained with ethyl methane sulfonate (EMS) of concentration of 6.00 mg with exposure time of one hour. Similarly, nine physical mutant strains were developed by exposure of spore suspension of parent strain to UV irradiation for a period of 60.00 min. Formation of zone of casein clearance of 72 mm with UV mutant strain, UV_4, 85mm with chemical mutant strains EMS_4 and EMS_6 and of 50 mm with parent strain were obtained. Total, twenty mutant strains were used for production of acid protease in submerged and solid state fermentation. Maximum acid protease activity (8.096U ml^{-1}) was noticed physical mutant strain UV_9 in submerged fermentation whereas in solid state fermentation, peak acid protease activity was achieved with EMS_{11} (1094.5 ± 6.11 U g^{-1}) followed by EMS_4, EMS_6 and UV_4. But there no specific relation was drawn from caseinolysis and acid protease production. In solid state fermentation, 1.53 fold higher acid protease activity was achieved with chemical mutant EMS_{11} over wild type with the optimized media of parent strain. Similarly physical mutant UV_9 also showed the enhanced protease activity by 2.01 fold than wild type under submerged fermentation.

Keywords: Acid protease, Mutation, *Aspergillus niger*, submerged and solid state fermentation

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INTRODUCTION

Proteases are hydrolytic enzymes which catalyze the cleavage of peptide bonds in other proteins into smaller peptide fragments [1]. These enzymes account to 60 percent of total world enzyme market [2] and are ubiquitous in nature thus occur in all living things such as plant, animal, microorganisms[3]. Extraction of proteases from plant and animal sources require many steps and also results in low enzyme yield. Hence microbial source is preferred because of their rapid growth and vulnerable to genetic manipulation [4,5]. Moreover, fungal strains offer more advantages of utilization of low cost materials, high protease productivity and easy in the recovery of enzyme [6]. Proteases are classified as alkaline, acid and neutral proteases according to the range of pH at which they exhibit

maximum activity. Microbial alkaline proteases have studied extensively than acid proteases. Fungal acid proteases find applications in the preparation of digestive syrup, soy protein digestion during sauce preparation, meat tenderization, hydrolyzing the gluten from wheat dough used for preparing biscuits in bakery making them crispy, in manufacture of alcohol for grain fermentation [7]. Among fungal strains, acid proteases from *Aspergillus oryzae* have well characterized compared to that of *Aspergillus niger*.

In view of biotechnological importance of acid protease, the present investigation was performed to enhance the acid protease activity, wild type *A. niger* was mutated genetically by conventional approach viz. physical and chemical methods. Further, the production of enzyme was investigated by both wild type and mutant strains in both Submerged Fermentation (SmF) and Solid State Fermentation (SSF).

MATERIALS AND METHODS

The mesophilic fungal culture was isolated from soil contaminated with abattoir waste collected from rural areas of Tirupati, A.P. India. The isolated fungal culture was identified as *Aspergillus niger* based on its morphological and microscopic characteristics and these values matched with values in standard reference book compendium of soil fungi [8]. Further the culture was screened on casein agar medium for protease production. The fungal inoculum was prepared by addition of 10ml of 0.1% triton X-100 solution to the 7^{th} day old slant and was shaken well to obtain homogeneous spore suspension.

(i) Physical mutation by UV irradiation [5]:
Various serial dilutions of fungal suspension were prepared and dilution 10^{-8}, 10^{-9} and 10^{-10} were distributed into sterilized petri plates (2 ml in each plate). These were exposed to UV radiations for varying time periods ranging from 5 to 120 minutes in UV chamber keeping the distance of UV source at 15cm. After UV radiation they were kept in dark for stabilization of thymine-thymine (T-T) dimmers. Parent type and UV treated fungal spore suspensions of 0.1 ml was inoculated into 25ml petri plate containing potato dextrose agar medium. Then developed fungal strains whose survival rate was less than 1% were screened on 1%casein and 2% agar for proteolytic actvity.

(ii) Mutation by EMS (Ethyl methane sulfonate) [9,10]:
In order to develop mutant fungal strains with EMS, eight test tubes with two millimeter of cell suspension (10^6) each were taken and one of them was kept aside as control and rest of them were incubated with EMS concentrations varying from 2mg to 10mg for 30 and 60 minutes time periods at room temperature (32 ± 2^0C) . After required period of treatment, the cells were centrifuged at 3000rpm for 10min at 4^0C, washed with sterilized phosphate buffer (pH 7.0) twice. A volume of 0.1 ml of EMS - treated fungal suspension was poured into sterilized petri plates containing potato dextrose agar medium. At the end of 7^{th} day, morphological changes of fungal suspension were noticed.

Significant morphological changes were found in spore suspension exposure to EMS concentration of 6mg for one hour incubation and then suspension was diluted. The plates having less than 1.00 % of survival rate over the control (without chemical treatment) were selected for screening of hyper-proteolytic mutants on casein – agar plates.

(iii) Production and comparison of acid protease by wild type and mutant strains in SmF [11]:
Acid protease enzyme production was carried out with both wild and mutant strains in SmF with molasses and cheese whey as the fermentation medium at temperature of 32 ± 2^0C. At the end of fifth day of incubation period, the fermentation broth was filtered and the filtrate was assayed for acid protease activity and protein content.

(iv) Production and comparison of acid protease by wild type and mutant strains in SSF [12]:
The fermentation medium (wheat rawa: 10 g; chickpea meal: 1.0% (w/w); dried potato peel: 1.0% (w/w)) was moistened with 60% salt mineral solution. Later the flasks were autoclaved, inoculated and incubated at a temperature 32 ± 2^0C for five days. Then acid protease was extracted with 50ml distilled water from the fermented solids and the contents were filtered with Whatman No.1 filter paper. The culture filtrate was used for enzyme assay.

(v) Analytical techniques:
Acid protease activity, concentrations of biomass and protein were estimated.

(vi) Protease activity assay [13, 14]
For protease assay following protocol was employed. In this, 3.0 ml of 0.6% (w/v) casein solution in 0.05M Tris - HCl (pH 6.5), 0.5 ml of the crude enzyme solution was added and the mixture was incubated for 10 min at room temperature. 3 ml of 0.44M TCA was admixed and the precipitate was removed by filtration, after standing for 30 min. Then, one ml of the filtrate was taken in a test tube, 2.5 ml of 0.55M Na_2CO_3 and 1 ml Folin -Ciocalteau reagent (diluted 2 times) were added. The color was developed for 30 min and absorbance was measured at 660 nm using UV/Vis-spectrophotometer (1700). The control was run in a similar manner except that TCA was added before the addition of enzyme solution. A reagent blank was prepared by adding 3.0 ml of Na_2CO_3 and 1 ml of Folin-Ciocalteau reagent (diluted 2 times) to 1ml distilled water. One unit of protease (U) can be defined as the amount of enzyme that releases 1 µ mole tyrosine/min under the reaction conditions for SmF. For SSF, a unit of protease activity (U) was defined as the amount of enzyme liberating µg tyrosine per ml of enzyme per min of incubation time. The protease activity was reported enzyme unit per gram of solid substrate, (U g^{-1}). Both fermentations were conducted in duplicate in 250ml Erlenmeyer flasks and the results were the mean and standard deviation of two trials.

(vii) Protein estimation [15]**:**
Protein content in the fungal filtrate was determined using bovine serum albumin as standard.

(viii) Mycelia dry weight [16]**:**
For this purpose, the fermented broth was filtered using preweighed Whatman No.1 filter paper. It was washed with water thrice and then dried at 105 ^{0}C over night in a hot air oven and weighed.

RESULTS AND DISCUSSION

Selection of a suitable strain capable of producing both amylase and protease together with commercially acceptable yield is a crucial step for bioprocessing[17]. Recent approaches for increasing protease yield include screening for hyper-producing strains, cloning and overexpression, fed-batch, chemostat fermentation, and optimization of the fermentation conditions **[18].** Mutation of industrially important microorganisms is imporatnt for the successful development of the various strains required in the production of various bio-products [5]. Development of mutant strains either by using conventional methods or by rDNA technology has played an important role in the enhancement of enzyme yield under the optimized conditions [19]. In the present investigation, conventional approaches of mutation such as physical and chemical methods were adopted to develop mutant strains of *A. niger* for enhanced production of protease. Fungal strain without mutation was depicted in Fig.I.

Fig.I. Growth of wild type *Aspergillus niger*

Spore suspension of wild type *A.niger* was treated with physical mutagen, UV irradiation, with various exposure times (0 to 120 min) to develop mutant strains. The survival data obtained on UV treatment with exposure time of 60.00 min was presented in Tab.I.

Tab.I. Experimental data on survival rate of physical and chemical mutants

Method	Control	Test	Survival rate
EMS (6 mg)	3980	30	0.750%
UV irradiation	4627	46	0.994%

** Data is represented as mean of two trials*

Survival rate = (Number of test colonies/ No. of control colonies) X 100

Lowering of survival rate of fungal spore suspension was noticed with increased exposure timings (greater than 1.00 h). Similar trend has been reported by some other investigators [2,7,14]. The survival rate of 0.994% was obtained with UV treatment of spore suspension of *A. niger*. From UV treated plates, a total of nine mutant strains were selected for screening on casein-agar plates. Fig.II. has revealed that maximum rate of caseinolysis was obtained with UV_4 and UV_7 (Zone of casein clearance 75 mm and 68 mm) whereas the minimum was observed with UV_6.

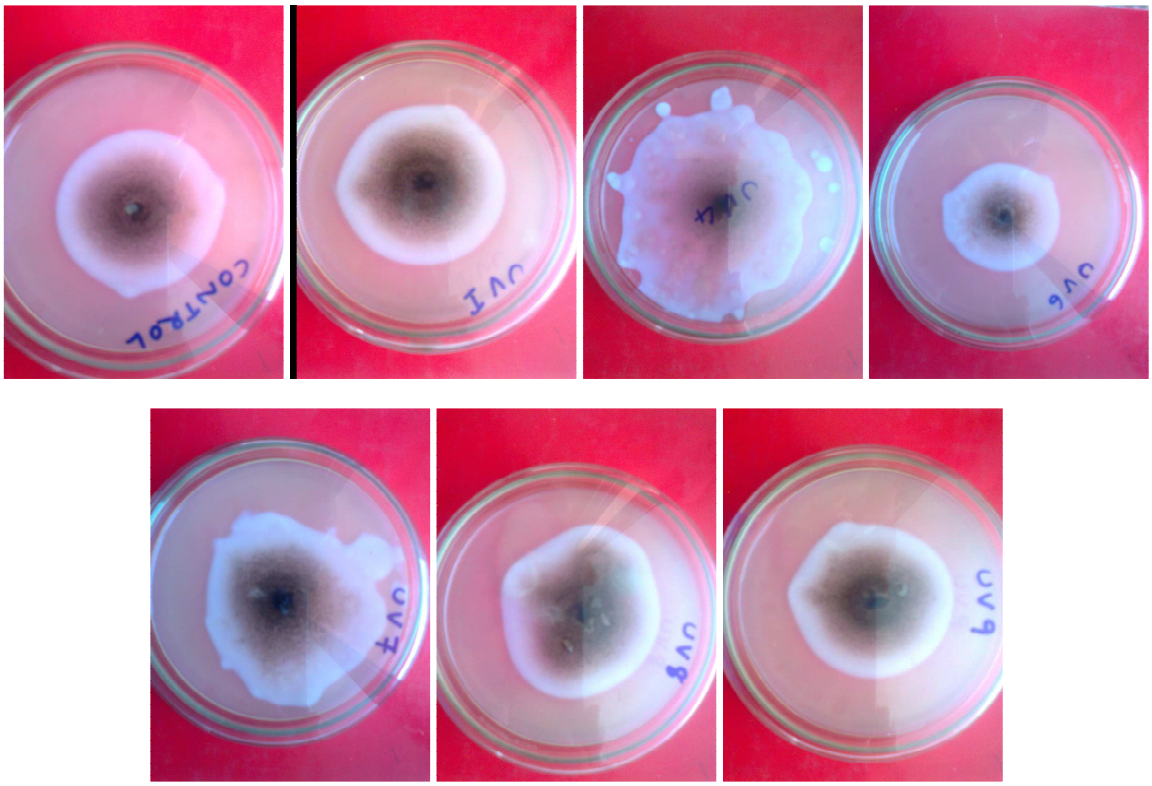


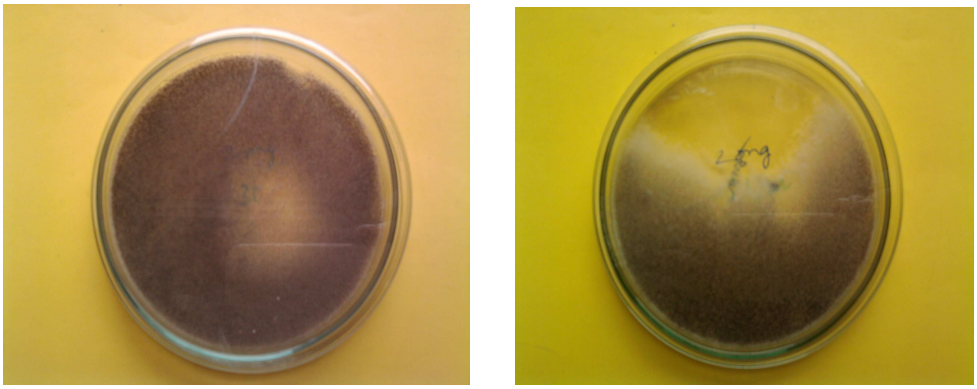
Fig.II. Caseinolysis by UV mutant strains (Time of exposure: 1 hour)

All the physical mutant strains could able to secrete acid protease with the formation of clearance of casein on casein – agar media plates and results were summarized in Tab.II.

Tab.II.Casein degradation by UV mutant strains

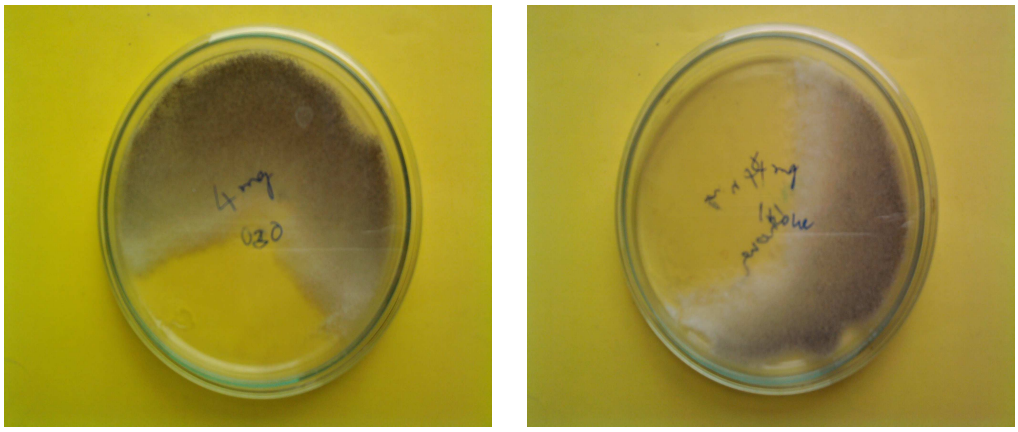
S. No.	Strain	Zone of casein clearance (mm)
1	Control	50
2	UV_1	58
3	UV_2	55
4	UV_3	52
5	UV_4	75
6	UV_5	51
7	UV_6	42
8	UV_7	68
9	UV_8	60
10	UV_9	54

Plate screening method had indicated that UV mutant strains except UV_6 were able to secrete more amount of extracellular protease than parent strain. Formation of zone of clearance of casein of 44 mm was reported with UV mutant [10]. Through this study, hyper proteolytic UV strains were developed.



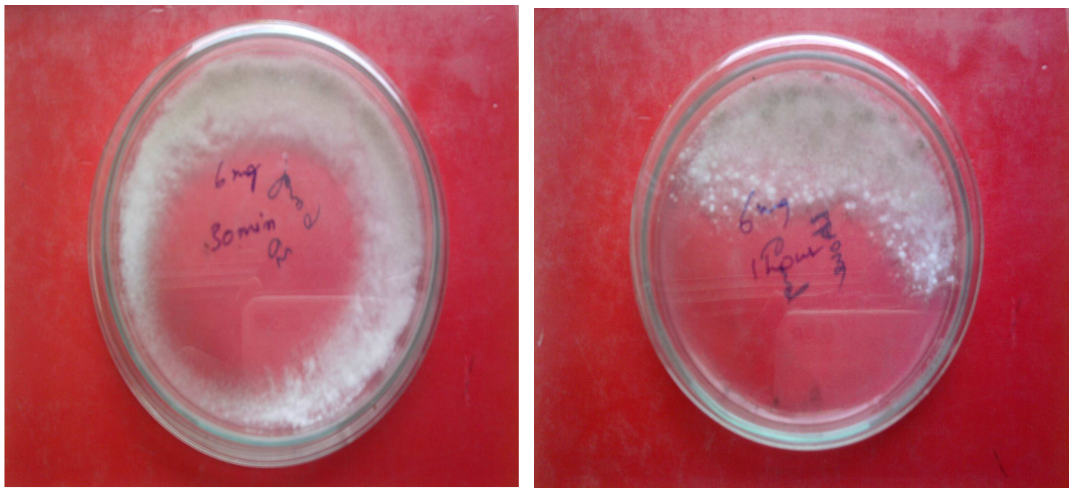
(a) **(b)**

Fig.III.Spore suspension in plates with 2mg EMS for (a) 30 and (b) 60 minute incubation



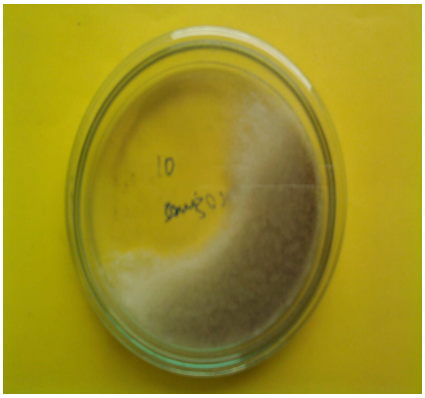
(a) **(b)**

Fig.IV.Spore suspension in plates with 4mg EMS for (a) 30 and (b) 60 minute incubation



(a) **(b)**

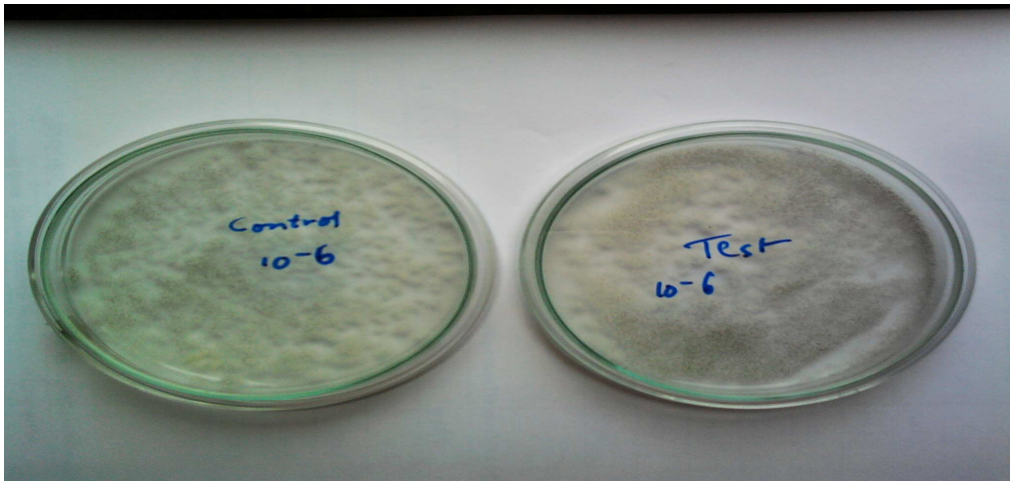
Fig.V.Spore suspension in plates with 6mg EMS for (a) 30 and (b) 60 minute incubation

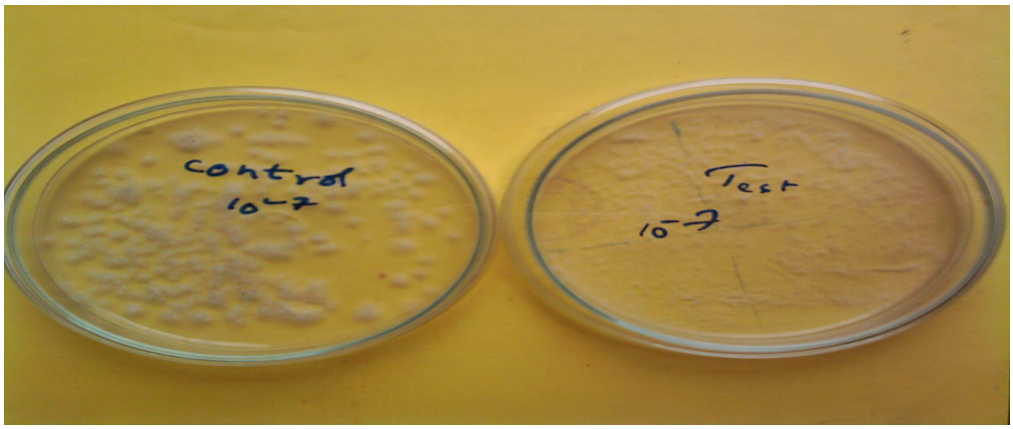


FigVI.Spore suspension in plates with 10mg EMS for 30 minute incubation

2. Effect of EMS on survivability of *A. niger*

The effect of various concentrations of EMS (0 – 10 mg) on wild type fungal isolate was depicted in Fig.III- VI. From these results, it was observed that EMS concentration of 2mg could not change the morphology of parent *A.niger* (Fig.III.) whereas significant morphological were found with spore suspension exposed to EMS concentration of 6.00 mg with one hour incubation (Fig.V).





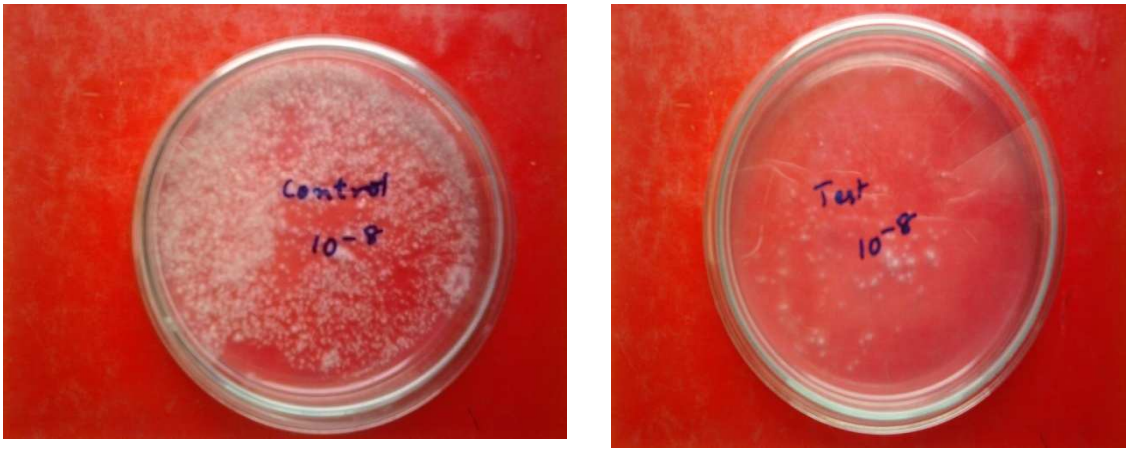
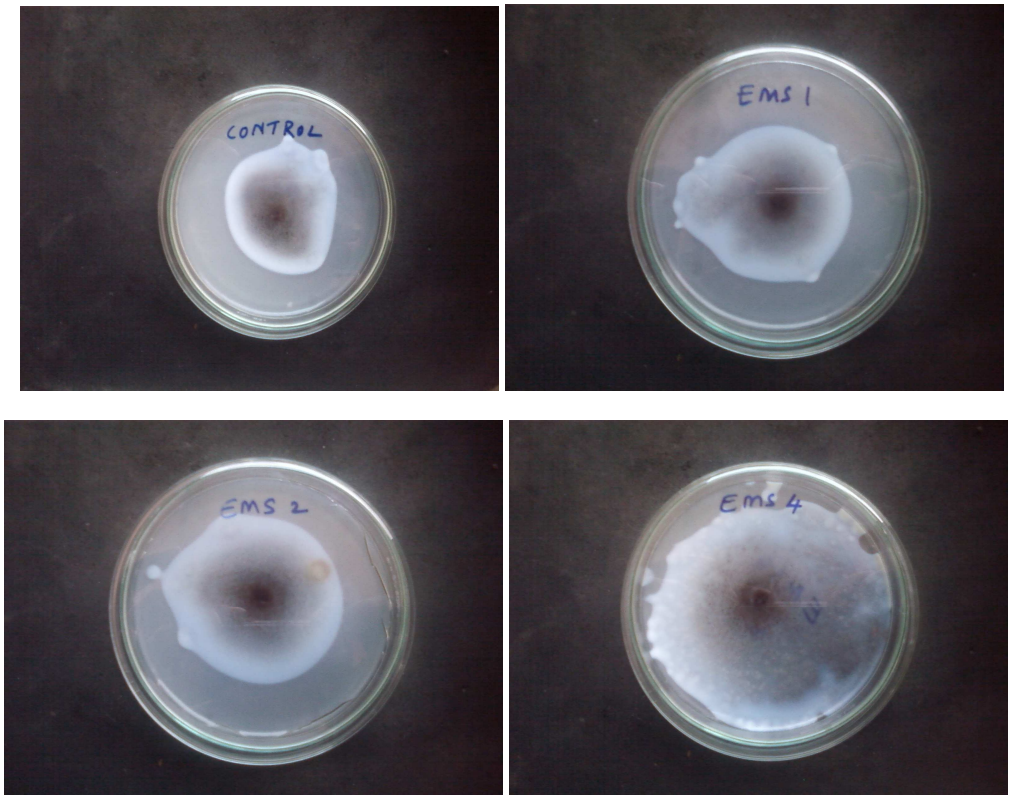


Fig.VII.Comparison of colonies of parent and chemical mutant strains

Then fungal suspension incubated with 6.00 mg of EMS for one hour was diluted (Fig.VII) and the survival rate of 0.75% was obtained with 10^{-8} dilutions. Mutagenic treatment affected the shapes and sizes of the mutant colonies [10].

The plates having minimum percentage of survival rate (Tab.I) over the control (without treatment) were selected for screening of hyper-proteolytic mutants on casein – agar plates and zones of caseinolysis were measured (Fig.VIII). From the results it was observed all the selected mutant strains were able to secrete more extracellular protease than wild strain and the maximum proteolytic activity (zone of clearance 85mm) was found with EMS_6 and EMS_4 mutant strains.



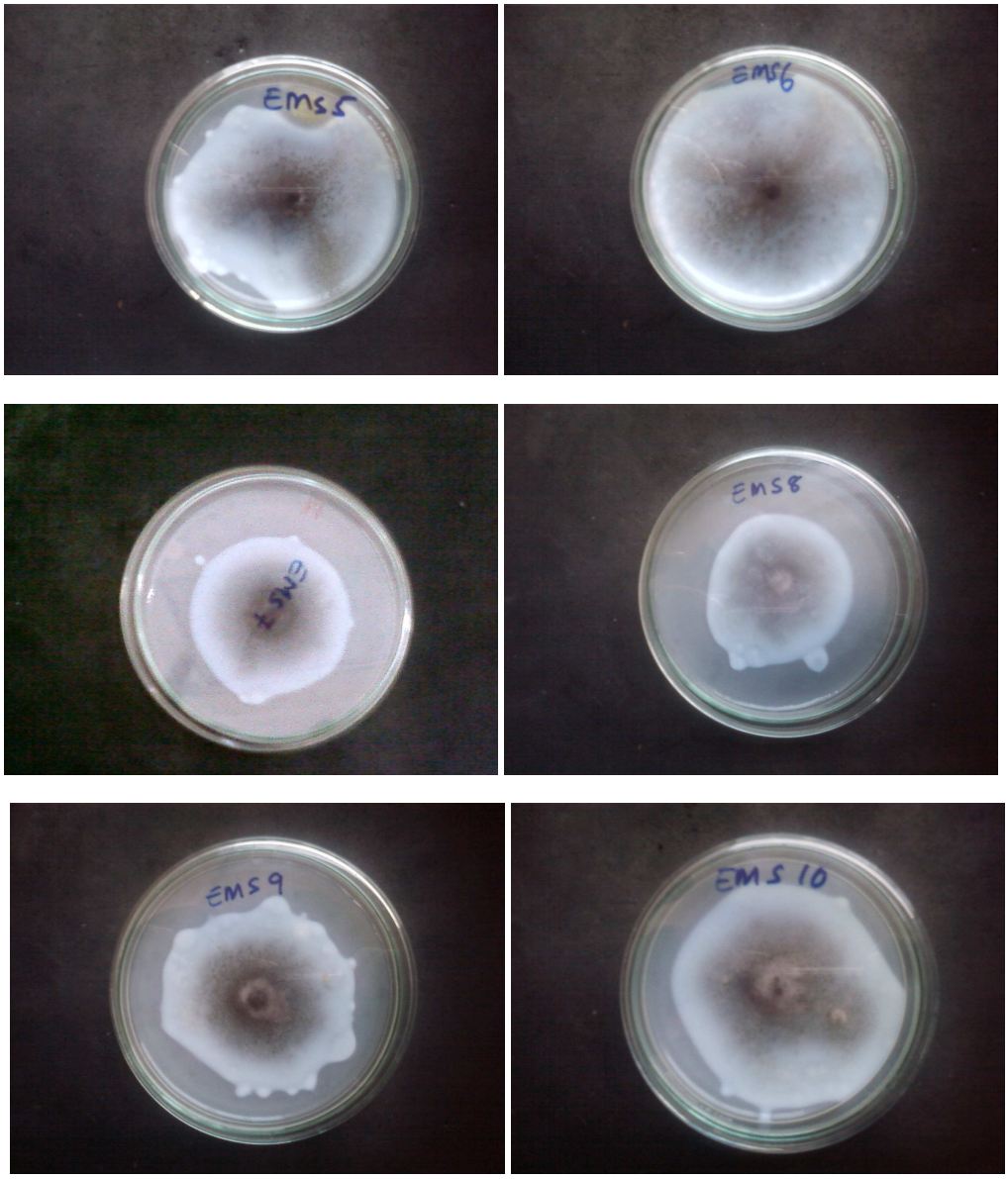


Fig.VII.The zones of clearance on casein agar plates produced by wild strain *A. niger* and its various chemical mutants after incubation of 72h at room temperature

The zone of casein clearance by both the wild type and chemical mutant strains was shown in Tab.III. When compared with previous literature and UV strains of present study, EMS4 and EMS6 have shown the maximum rate of caseinolysis.

From the above results of mutational studies, it was noticed that EMS induced mutant strains (EMS_4 & EMS_6) were more effective than UV irradiated strains (UV_4 & UV_6) by plate screening method.

Tab.III. Results of casein clearance by wild and mutant *A. niger*

S.No.	Fungal strain	Zone of casein clearance (mm)
1	Control	49
2	EMS_1	53
3	EMS_2	56
4	EMS_3	50
5	EMS_4	85
6	EMS_5	68
7	EMS_6	86
8	EMS_7	56
9	EMS_8	54
10	EMS_9	64
11	EMS_{10}	60
12	EMS_{11}	70

**Values in the table represented as mean of two trials*

3. Acid protease production by wild and mutant strains of *A.niger* through SmF

UV irradiated and EMS-induced mutant strains were further evaluated through shake flask enzyme production with preoptimized medium components and growth conditions of parent strain of *A. niger* [11]. Among the developed UV mutant fungal strains, UV_3, UV_4 were equally effective strains as control strain where as higher acid protease activity was achieved with UV_9 (Fig.IX). Enzyme productivity by the developed chemical mutant strains was shown in Fig.X and it was observed that EMS_2, EMS_4, EMS_5 and EMS_6 could secrete enhanced amounts of acid protease with unoptimized fermentation medium. Maximum enzyme productivity of 7.12U ml^{-1} was obtained with EMS_4. However, it was noticed that enhanced protease production was observed with physical mutant fungal strain rather than EMS-induced strains. The above results confirmed that UV_9 was the hyper-proteolytic fungal strain with 2.12 increase fold in acid protease activity.

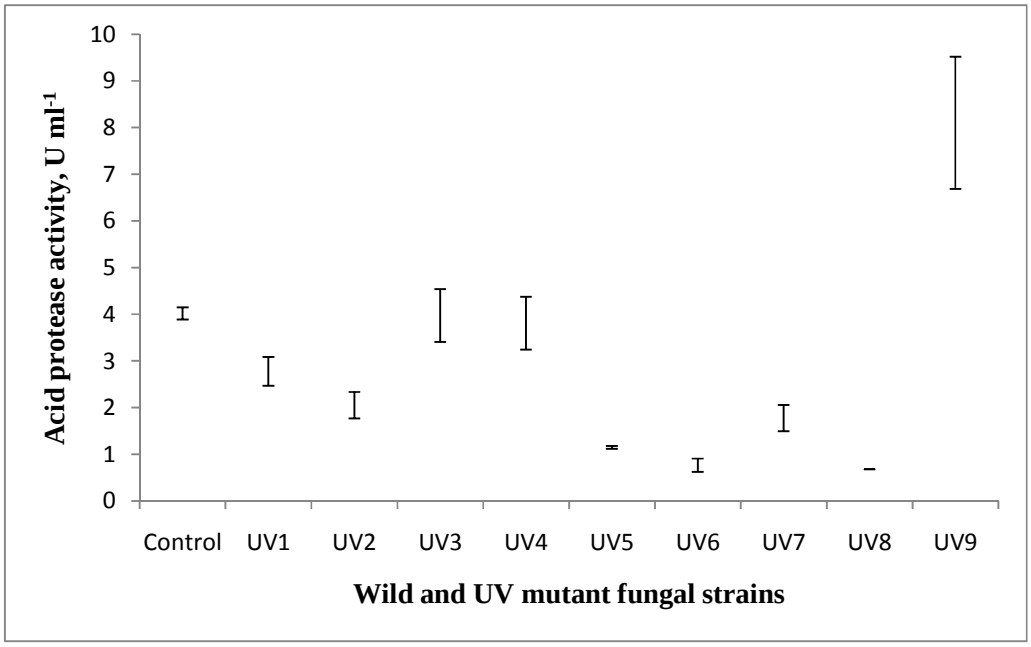


Fig.IX.Protease production with wild and physical mutant strains in SmF

** Values in graph are represented as mean ±SD (Standard deviation)*

Published literature indicated that the optimized conditions for protease production by wild and UV-mutant strains of *Pseudomonas* sps separately and reported that suitable temperature and pH of wild and mutant strains were not the same, finally achieved 2.5 fold increased production with mutant Strain[5].

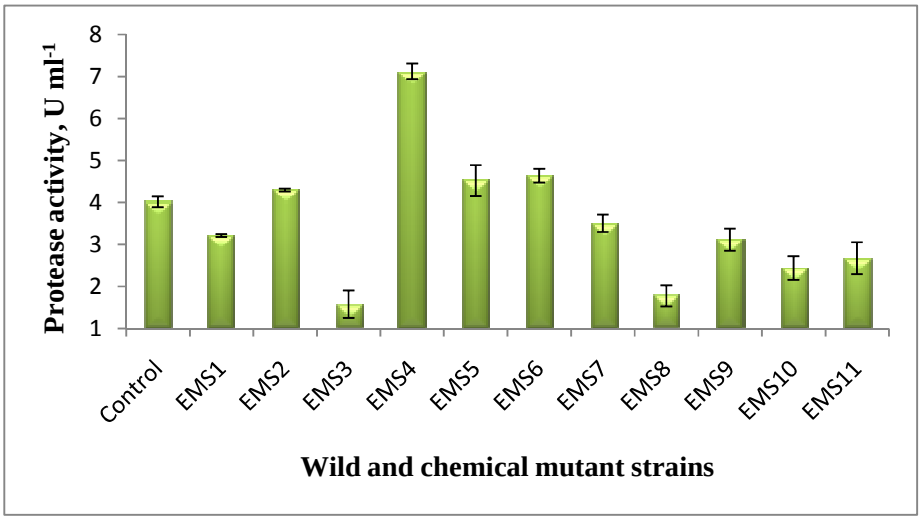


Fig.X.Protease production with wild and chemical mutant strains in SmF
** Values in graph are represented as mean ±SD (Standard deviation)*

4. Acid protease production by wild and mutant strains of *A. niger* through SSF

Extracellular protease production was performed using the both wild and developed mutant strains in SSF and results were depicted in Fig XI & Fig XII. Enhanced acid protease activity was achieved with EMS_{11} (1094.32 ± 6.11 U g^{-1}) followed by EMS_4 (1083.81 ± 7.96 U g^{-1}), EMS_6 (978.24 ± 5.06 U g^{-1}) and UV_4 (880.57 ± 43.93 U g^{-1}). Physical mutant, UV_7, could yield an enzyme activity of 756.53 ± 45.96 U g^{-1} which was approximately 93.56 % of that of parent strain. Very low enzyme activities were noticed with MCH_3 (196.56 ± 16.22 U g^{-1}) and UV_3 (175.08 ± 14.99 U g^{-1}). Remaining all mutant strains were not as effective as wild type strain.

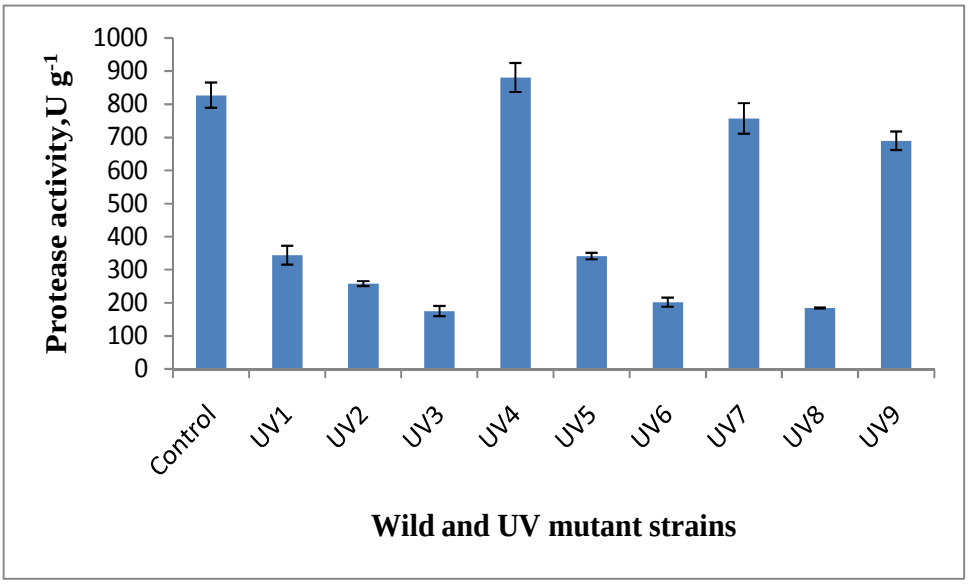


Fig.XI.Protease production with wild and physical mutant strains in SSF
**Values in graph are represented as mean ±SD*

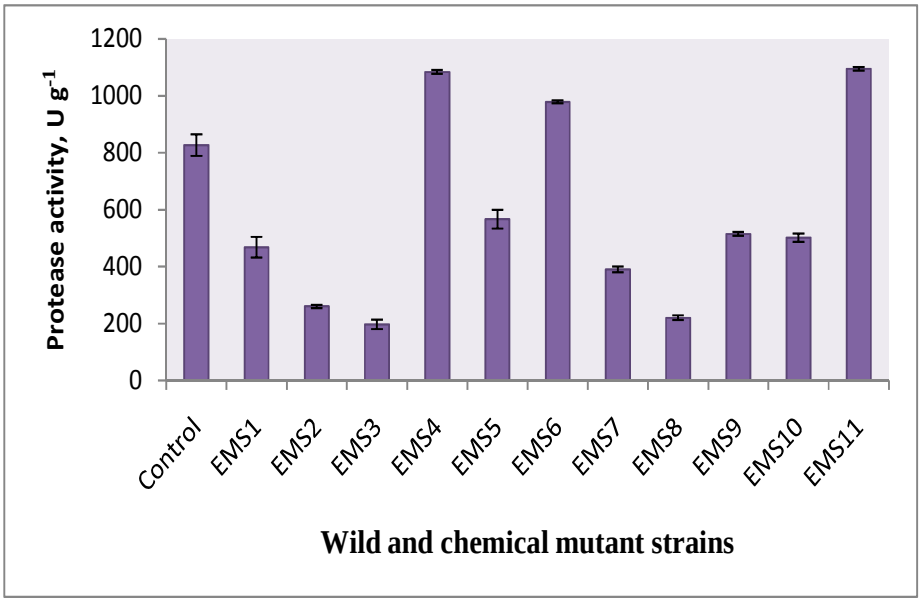


Fig.XII.Protease production with wild and chemical mutant strains in SSF
** Values in figure are represented as mean ±SD*

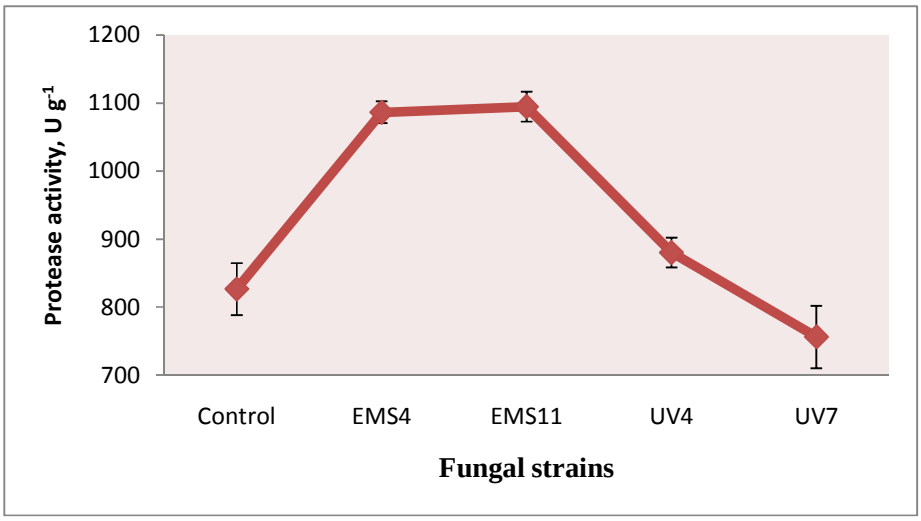


Fig.XIII.Comparison of protease production by wild, physical and chemical mutants in SSF
**Values in graph are represented as mean ±SD*

Comparative productivities of acid protease by parent, chemical and physical mutants in SSF were shown in Fig.XIII. From this, EMS_{11} is able to secrete significant amount of protease as compared wild *A.niger*.

A combination of two mutational agents – ethyleneimine and the UV rays were used by for acid protease production from *Penicillium* sps [20]. Most of the developed UV and EMS- induced strains were not able to yield more amount of extracellular acid protease since both SmF and SSF were performed with the optimized media of wild type of *A. niger*. Moreover, it was noticed that there was no exact correlation between casein clearance and acid protease activity through SmF and SSF.

CONCLUSION

In this study both physical such as UV irradiation, and chemical, EMS were employed on a local isolate *A.niger* to obtain mutant strains. Both of these treatments affected the morphological and genetically of fungal mutant strains. EMS treated strain, EMS_4, showed the highest zone of casein clearance. These mutant strains were enhanced the production of acid protease on SSF and SmF. In solid state fermentation, 1.53 fold higher acid protease activities were achieved with chemical mutant EMS_{11} over the wild fungal strain. Similarly physical mutant UV_9 also enhanced the protease production by 2.01 fold than the wild strain under submerged fermentation.

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