Research Article

Isolation of Novel Mutant Strain for Enhanced Production of Extracellular Serratiopeptidase from Mangrove Soil

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ABSTRACT

Improvement of microbial strains for the over production of industrial products has been the characteristic of all commercial fermentation processes. It owes much of its utility as an empirical approach to the existence of rate limiting catalytic steps within all metabolic pathways. The purpose of the present investigation is to enhance production of the Serratiopeptidase and to study the effect of physical and chemical mutagenesis. A total of 24 actinomycete strains isolated from mangrove soil sediment were used and found to grow well in medium. Four of the isolated strains exhibited Serratiopeptidase activity. KMFGS13 identified as *Streptomyces* sp., was found to be an excellent producer for Serratiopeptidase activity in submerged fermentation. Therefore, this strain was selected and subjected to mutations by Ultraviolet (UV) light followed by nitrous acid (HNO_2) treatment for enhanced activity. UV-7 mutant exhibited 33.9% higher activity than the wild strain in UV treatment. Whereas in chemical mutagenesis, the UV-N7 mutant showed maximum activity by 60.1% higher than wild strain. Biomass growth was also found to be high in nitrous acid treated strains. Hence, these results signify that UV and HNO_2 were effective mutagenic agents for strain improvement.

Keywords: Induced Mutagenesis, Serratiopeptidase, Strain Improvement.

INTRODUCTION

Microbial enzymes are frequently useful than enzymes derived from animals or plants.[1,2] Because of the great variety of catalytic activities and the high yields possible as well as ease of genetic manipulation but also its regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media, thermolabile in character & specific in their action. These are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer.[3,4] Serratiopeptidase [EC 3.4.24.40] is an extra cellular metalloprotease enzyme and potent analgesic and natural anti-inflammatory drug.[5,6] It was known to produce by submerged fermentation by various bacterial strains of *S.marcescens*. The bacterium has been well extensively reported for the production of Serratiopeptidase enzyme. The exponential increase in the application of Serratiopeptidase in diverse fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement. Quantitative enhancement requires strain improvement and medium optimization for the overproduction of the enzyme, as the quantities produced by culture strains are low. Thus, it is essential to improve the strain continuously to make the fermentation process economically successful. Developed strains can reduce the costs with increased productivity and can possess some specialized desirable characteristics. Such improved strains can be obtained by mutation so that better strains are successfully picked out and further improved.[7,8] A number of previous studies dedicated to the optimization of the nutritive medium composition and the cultivation conditions for various enzyme productions in different organisms. But only some considered the possibilities for enhancement of enzyme production by strain improvement.[9,10] However, there were no reports found on Serratiopeptidase production from actinomycetes. It is evident that mutagenic treatment of actinomycete spores with UV light and nitrous acid (HNO_2) can be beneficial in increasing the yield of their metabolites for the development of effective technologies for industrial scale production of Serratiopeptidase.

In the present study, enhancement of serratiopeptidase productivity of actinomycete strains by induced mutagenesis of both physical (UV) and chemical (HNO_2) mutagens were employed in systemic manner to obtain mutants that gave higher Serratiopeptidase production. For UV irradiation, method of Parekh *et al*., 2000[11] was adopted. For chemical mutagenesis using HNO_2 method of Delic *et al.,* 1970[12] was followed. The parent strain *KMFGS13* was treated with mutagens UV and HNO_2 consecutively.

MATERIALS AND METHODS

Sample Collection

The soil samples subjected to production of Serratiopeptidase were isolated from mangrove soil sediment collected at Koringa mangrove forest, Kakinada region, Andhra Pradesh, India.

Screening of Microorganisms

After collection of the samples in sterile container, the sample was brought to the laboratory. Thereafter, the dominant organisms were isolated and individually streaked on agar plates. The Strain of KMFGS13 was

identified for the best for the production of serratiopeptidase enzyme. The isolation medium consisted of modified production medium - IV was found to be best. The strains were maintained on YEME slants and stored at 4°C.

Identification of Strain

Morphological, cultural characteristics as well as physiological properties of strain KMFGS13 of the isolate have been studied for the identification according to the methods described by Shirling and Gottlieb.[13] Using the trinoccular microscope [Labo America Inc., USA][14] and a scanning electron microscope S-570, Hitachi[15], the detailed observation of mycelial and spore morphologies was performed and chemical analysis of cell wall was also performed with the methodology of Becker *et. al.*,[16] then Screening for prospective Serratiopeptidase producing strains was carried out by casein hydrolysis supplemented with 1mM PMSF and caseinolytic activity on the basis of the coefficient K as shown in the Table 1, which reflects the ratio between the diameter of the clear halo around the colony (d_H, mm) and the diameter of the respective colony (d_C, mm), measured on the 5th day, at 28°C ($K=d_H/d_C$) then followed by specific assay for Serratiopeptidase activity mentioned in Indian Pharmacopeia 2010.[17]

Table 1: Screening of prospective Serratiopeptidase producing actinomycete isolate

Actinomycete strain	k	Serratiopeptidase activity(U/ml)
KMFGS11	1.4	42.81
KMFGS12	1.2	38.15
KMFGS13	**5.3**	**88.32**
KMFGS14	1.9	61.94

Production of Serratiopeptidase

First, inoculum was prepared for production medium containing collected sample slant culture of strain KMFGS13 on modified production medium - IV medium supplemented with 10% yeast extract and malt extract medium was inoculated into 45 ml of the medium consisting of pH range 7.0±0.2 before autoclaving and incubated at 27°C for 72 hours on a rotary shaker with 200 rpm. Next, for the extraction of Serratiopeptidase, 10% of the culture was transferred to 45 ml of the fresh production medium and incubated for 96 hr in the same way as the above. After fermentation, the medium was centrifuged at 6,000 rpm for 20 minutes at 4˚C to remove the biomass and cell debris. As the enzyme produced is extracellular, the supernatant was collected and assay of Serratiopeptidase was done. The strain which showed efficient Serratiopeptidase activity was further selected to study the effect of mutation on their enzyme production.

Serratiopeptidase Assay

Enzyme activity was assayed in the culture filtrate was determined by IP 2010[17], one unit of Serratiopeptidase is defined as the amount of enzyme required to liberate one µm of free tyrosine per minute under the specified assay conditions. The tyrosine units are dissolved in sodium carbonate solution, which is alkaline in nature. The *Folin'sciocalteau reagent* helps in color development where the tyrosine units bind to the copper molecule in the reagent and causes the reduction of phosphor molybdate which is present in the reagent. There is formation of tyrosine-copper molybdate colored complex. The intensity of color depends upon the tyrosine units present which are read at 660nm.

Conditions for UV mutagenesis

Spore suspension containing 0.5× 10^2 ml obtained from 7 days old culture of parental strain cultivated on YEME slant was UV treated and the UV lamp 220 V, 40 W, 50 Hz was used as a source of UV light. This was carried out at a distance of 26.5 cm from the center of the UV lamp. The exposure time was 0, 30, 60, 90, 120, 150, 180, 240 and 360 sec respectively through gentle shaking and the samples were stored at 4°c overnight in a dark place to avoid photo reaction. And followed day pour plating technique done for the serial dilution of the samples till 10^{-7}. After that 0.1ml of samples were inoculated. Then the Petridishes were cultivated at 28°C for 7 days to count the number of survived colonies and the survival curves were drawn. Screening of prospective mutant strains was carried out on the basis of the coefficient K, and assay for specific activity done as described above. The screened mutant strains were isolated as pure cultures on YEME and stored at 4°C before further experiments.

Conditions for HNO_2 mutagenesis

The spore suspension containing 0.5× 10^2 ml obtained from 7 days old culture of UV treated strain cultivated on YEME was also treated with HNO_2 dissolved in 0.05M acetate buffer (pH 5.0) at concentrations of 100µg/ml for 6h. The mutagenic effect of HNO_2 was eliminated by adding 0.01M sodium nitrite. At regular intervals, 0.1 ml was taken and the samples were seeded onto the surface of Petridishes containing YEME agar. Screening of prospective mutant stains was carried out as listed above.

The cell suspension of the parent strain was prepared by using buffer pH-5. To 9 ml of the cell suspension in buffer 1ml sterile stock solution of 0.01M sodium nitrite was added. Samples of 4ml are withdrawn at regular intervals of 0, 30, 60, 90, 150, 180, 240 and 360 sec. Each of 1ml samples was neutralized with 0.5ml of 0.1 M NaOH serially diluted and plated on YEME medium. The dose-survival curve was constructed after nitrous acid treatment. Plates having survival rate between 15 and 1 % were selected for the isolation of mutants. The stable mutants were selected based on consistent expression of the phenotypic character up to three generations and maintained on YEME slants. The plates were incubated at 28°C for 7 days.

Conditions for submerged production of extracellular Serratiopeptidase

A nutritive medium of a following composition (g/L): Soyabean meal- 15 g, Glucose-15 g,- Glycerol-2.4 ml, NaCl-4.5 g, $CaCO_3$-0.9 g, tryptone-15.3 g, KH_2PO_4-2.24 g, distilled water up to 1 L, pH 7.0±0.2 was used for the submerged Serratiopeptidase production. The nutritive medium was sterilized at 121°C for 15 min. The cultivation was carried out in 250 ml Erlenmeyer flasks, containing 50ml nutritive medium on a rotary shaker at 200 rpm, 27±1°C for 96 hrs. The nutritive medium was inoculated with 10% spore inoculum. After the cultivation, the biomass was separated through filtration and the cell free cultural broth was used as crude enzyme. The amount of the biomass was determined gravimetrically by drying the samples to constant weight at 105°C.

RESULTS AND DISCUSSION

Out of total 24 actinomycete strains, four prospective strains isolated from mangrove soil sediments for the production of extracellular Serratiopeptidase were screened and activity of all strains was also determined in submerged fermentation. Among the tested, KMFGS13 strain displayed the highest Serratiopeptidase activity with 88.32 IU/ml and was screened as parental strain for mutagenesis.

Usually, strain improvement of extracellular enzyme production is reached by induced mutagenesis with various mutagens, such as UV light, nitrous acid (HNO_2), applied separately or in combination.[9,19,20,22-24] The effectiveness of the induced mutagenesis depends on the type of mutagen, dose and duration of exposition. Survival curves after UV and HNO_2 treatment of KMFGS13 were drawn and analyzed as shown in Figure 1. All of the survived colonies were morphologically identical according to hyphal diameter, mycelium colour, diameter and colony shape, but only 25% of colonies demonstrated Serratiopeptidase activities higher than the parental one.

As identified by Hopwood *et. al.*,[18], 99.9% kill correlates with the highest frequency of mutations and highest possibility for isolation of mutants with increased enzyme productivity[9, 19&20]. The plates having less than 1% survival rates were used to select mutants. Induced mutagenesis by UV as seen in Figure 2 showed that the mutant strain UV7 demonstrated the highest Serratiopeptidase activity (133.8 IU/ml), which is 33.9% higher than the parental activity. The strain UV7 kept its Serratiopeptidase activity unchanged in 3 consecutive generations and was screened as the most prospective UV mutant for further investigations.

The UV mutant was selected and was subjected to further strain improvement by HNO_2 treatment. HNO_2 is considered to be very effective chemical mutagen. The selected HNO_2 as seen in Figure 3 treated isolates were obtained from plates having less than 15% survival rates. The achieved effect of mutagenesis on Serratiopeptidase activity (220.9 IU/ml) of KMFGS13 lower than those achieved by Ellaiah *et al.*, 2002[19] and almost equal with those achieved by Karanam and Medicherla.[20]

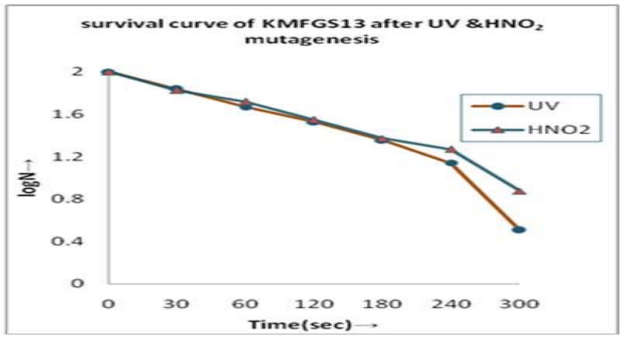

Figure 1: Survival curve of KMFGS13 after UV &HNO_2 mutagenesis

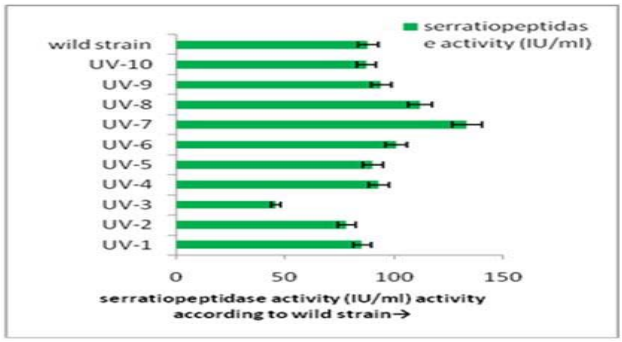

Figure 2: Influence of the UV mutagenesis on Serratiopeptidase activity of *KMFGS13*: wild strain; UV: UV induced wild strain.

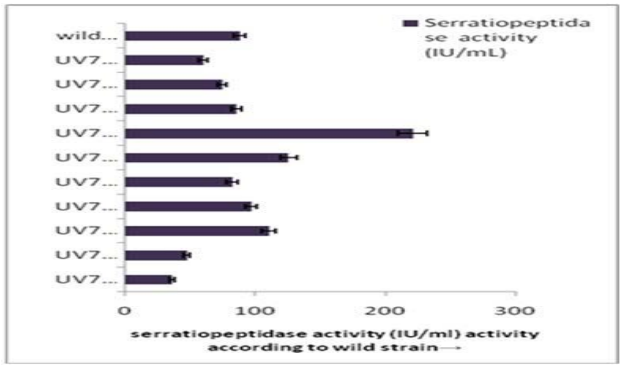

Figure 3: Influence of HNO_2 treatment on Serratiopeptidase activity of *KMFGS13*:- wild strain; UV7N: HNO_2 induced UV mutants.

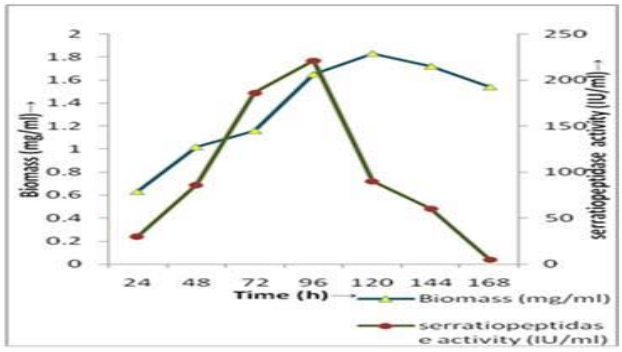

Figure 4: Dynamics of Serratiopeptidase production of UV7N-7 mutant.

Simultaneously, the biomass growth correlated with the enzyme activity as in Figure 4. Thus, overall 2.5 fold increase in activity achieved by induced mutagenesis.

CONCLUSION

The extracellular production of Serratiopeptidase is massive and rising. Wide-ranging and continual screening for new microorganisms and their enzymatic activity will unlock new, easy routes to resolve fermentation problems. The present work was undergone on actinomycete isolate KMFGS13 which was isolated earlier in our laboratory. The selected isolate was tested for initial screening and specific assay for the Serratiopeptidase activity as per IP 2010 by submerged production and then the strain was subjected to mutations by Ultraviolet (UV) light followed by Nitrous acid (HNO_2) treatment for enhanced activity. The results includes, through the UV mutagenesis that the UV-7 mutant exhibited 33.9% higher activity than the wild strain. Whereas upon nitrous acid treatment in chemical mutagenesis, the UV7N-7 mutant showed maximum activity by 60.1% higher than wild strain. Correspondingly, the biomass growth was also high in nitrous acid treated strains. Thus, these results indicate that UV and HNO_2 were effective mutagenic agents for strain improvement.

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