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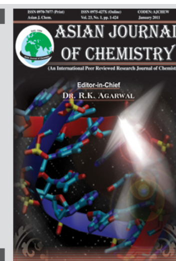

Mutation Induced Enhanced Production of Bioactive Metabolites by a Mutant Strain of Marine *Streptomyces rochei* (Isolate No.10)

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The selection of spontaneous and induced mutants in *Streptomyces* might generate enhanced producers of bioactive metabolites. In the present, investigation to enhance the production of biomedically important bioactive metabolites by subjecting the indigenous bioactive metabolites producing strain of *Streptomyces rochei* to improvement by natural selection and random mutagenesis UV and N-nitroso guanidine (NTG). The isolation of mutants and their antimicrobial activity of the selected mutants were described. The best natural selectant GRR_{NS3} showed 110 % higher antimicrobial activity than the wild strain. The bioactive metabolites yield of the best UV mutant GRR_{UV6} was 118 % higher than the parent strain (GRR_{NS3}) and 129 % higher than the wild strain. The bioactive metabolites yield of the best NTG mutant GRR_{NTG8} was 111 % higher than the parent strain (GRR_{UV6}) and 143 % higher than the wild strain. The results indicate that UV and N-nitroso guanidine were effective mutagenic agents for strain improvement of wild strain *Streptomyces rochei* for enhanced bioactive metabolites productivity.

Key Words: Bioactive metabolites, *Streptomyces rochei*, UV, N-Nitroso guanidine.

INTRODUCTION

The indispensable role of biotechnology is increasing in nearly every industry, including the health care, pharmaceutical, chemical, food and agricultural industries. Biotechnological production of small-volume high-value drugs, chemicals and bioproducts is well justified economically. However, production of large-volume low-value bioproducts requires the development of lower-cost and higher-yield processes. So, strain improvement is important in applied microbiological research, especially in the production of clinically important antibiotics. Towards this goal, improvement of microbial strains for the over production of industrial products has been the hall mark of the all industrial commercial fermentation processes and improved microorganisms have traditionally been developed through random mutagenesis followed by screening processes[1,2]. While of immense fundamental interest, insights into the regulation of antibiotic production provide new opportunities for knowledge-based approaches for strain improvement to complement the classical and undoubtedly successful strategy of mutation and screening for improved productivity.

In the recent years, marine microorganisms have been given attentions as a prodigious source of bioactive compounds with intriguing structure and interesting biological activity for drug development. The marine filamentous bacteria belong to the genus *Streptomyces* of *Actinomycetes*, are exceptionally rich source for a huge number of secondary metabolites. They are known as one of the most prospective natural sources for production of over two-thirds of known antibiotics[3-5]. The low level production is found in strains producing both natural and so called "hybrid" antibiotics (resulted from heterologous gene expression or gene knockout experiments). Due to the high frequency of phenotypes of low productivity and viability in *Streptomycetes*[6], a constant selection of stable populations is necessary. Improvement of production of clinically valuable antibiotics in *Strptomycetes* is of great economic importance. Great variety of gene and cellular engineering methods as well as random mutagenesis is used to increase the production of desired bioactive compounds. Current methods of antibiotic production, ranging from classical random approaches to metabolic engineering, are either costly or labour-intensive. The applications of modern techniques of direct genome manipulation frequently encounter serious impediments due to limited insight to the genetics, physiology and biochemistry of the producer organism[7]. Although mutation and random selection methods have been succeeded in generating many industrial strains, they are time-consuming and high-cost processes[2,8]. Induction of mutations is one of the applied techniques which used different types of physical and chemical

mutagens, either individually or in different combinations, doses and times[9-12].

The spectacular successes of strain improvement in industry are mostly attributed to the extensive application of mutation and selection. Successive cycles of mutation and selection have been widely used for the improvement of antibiotic yields in fungi and actinomycetes[13-15]. In this way large increases in productivity have been achieved through the stepwise accumulation of favourable mutations, many of which are individually of small effect[16,17].

The aim of the present investigation is to enhance bioactive metabolites of the wild strain *Streptomyces rochei* by subjecting it to improvement by natural selection and random mutagenesis by UV irradiation and N'-nitrosoguanidine (NTG) treatment.

EXPERIMENTAL

All reagents used were of analytical or extra-pure grade and were obtained from commercial sources, Himedia (Mumbai) and Merck Co., India.

Microorganism and cultural conditions: *Streptomyces rochei*, a wild strain, isolated from Bay of Bengal, Visakhapatnam coast and was identified according to Bergey's manual of Systematic Bacteriology 1985. Pure culture was maintained on starch casein agar (SCA) slants and stored in the refrigerator at 4 °C until further use. The mutants were cultivated in Trypticase Soy Broth (TSB)[18,19] supplemented with 30 days aged natural sea water and distilled water (50:50 %). For seed culture preparation, a loopful of mycelium and spores were taken from the slope culture and used to inoculate 250 mL flask containing 25 mL of TSB medium and then incubated at 30 °C for 2 days. Five mL of seed culture were used to inoculate 250 mL flask containing 50 mL of TSB medium and the cultivated flasks were further incubated for additional 5 days at 30 °C on the rotary shaker (110 rpm). The culture broth was then centrifuged at 10,000 rpm for 15 min and the clear supernatant broth was used as the crude bioactive metabolites source. After mutagenesis, the enhanced production of bioactive metabolites of selected mutants were compared with the wild-type strain on oat meal agar (OMA) medium[20] was prepared by boiling 40 g of oatmeal per 1 L with 30 days aged natural filtered sea water and distilled water (50:50 %) for 15 min, insoluble matter was removed by centrifuging 15 min at 10,000 rpm supernatant was kept and 2 % agar was added.

Test organisms: The selective human pathogenic microorganisms used for antimicrobial activity were fungi-*Candida albicana* (MTCC 183), gram positive bacteria - *Staphylococcus aureus* (MTCC 3160) and gram negative bacteria-*Escherichia coli* (MTCC 443). All the strains obtained from IMTECH, Chandigarh, India. Potato dextrose agar (PDA) and nutrient agar (NA) media were used for the cultivation of test fungi and bacterial species respectively and all the cultures were preserved at 4 ºC.

Isolation and selection of mutants

Natural selection: The wild strain *Streptomyces rochei* was subjected to natural selection. The organism grown on starch casein agar (SCA) slants was scraped off into physiological saline containing 0.85 % NaCl-0.1 % Tween-80[21] to give uniform suspension. Four mL of cell suspension (10^9 cells/mL) contained in a petri dish was transferred into a sterile conical flask and thoroughly shaken for 0.5 h on a rotary shaker to break the spore chains. The spore suspension was the filtered through a thin sterile cotton wad into a sterile tube, to remove vegetative mycelium from the suspension and the 1 mL spore suspension was then serially diluted with sterile physiological saline and spore suspension was spread for the single colony isolation to calculate the percentage survival. Samples with a death rate of 99.0 % were subjected to subsequent isolation.

One mL of the respective dilutions of spore suspension was added to the melted oat meal agar (OMA) medium at 40 °C and after thorough mixing was poured into sterile petri dishes. The plates were incubated at 30 °C for 10 days. Colonies were selected on the basis of the morphological variations including sporulation and the presence of aerial hyphae. The selected colonies were transferred onto oat meal agar slants and were allowed to grow at 30 °C for 10 days. A total of 9 isolates were selected and were designated as natural selectants (GRR_{NS1} to GRR_{NS9}). These selectants along with the wild strain were tested for the production of bioactive metabolites by agar well-diffusion method and their activity measure in the terms of inhibition zone diameter in mm.

Mutagenesis and mutant isolation

Physical mutagenesis by UV irradiation: Further work on improving the yield was done by mutation and selection. High yielded natural selectant was subjected to UV irradiation. The spore suspension of the best natural selectant (GRR_{NS3}) was prepared in sterile physiological saline following the same method mentioned in natural selection. Four mL volume of the spore suspension was pipetted aseptically into sterile petri dishes of 80 mm diameter having a flat bottom. The exposure to UV light was carried out in a "Dispensing-Cabinet" fitted with TUP 40W Germicidal lamp which has about 90 % of its radiation of 2540-2550 Å. The exposure was carried out at distance of 20.0 cm away from the centre of the Germicidal lamp (UV light source) with occasional shaking. The exposure times were 15, 30, 45, 60, 75 and 90 min. Each UV exposed spore suspension[22] was stored in dark overnight to avoid photo reactivation, which may compromises the efficacy of the mutagenic treatment. Then was serially diluted in sterile physiological saline and pour plated on oat meal agar 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. The growing colonies were transplanted on slants for further studies[23]. A total of 9 colonies (designated as GRR_{UV1} to GRR_{UV9}) were selected from the petri plates showing less than 1 % survival rate (45, 60 and 75 min UV exposure time) and tested for antagonistic activity. Among the selectants, the best UV mutant strain was used for further studies.

Chemical mutagenesis by NTG treatment: The best UV mutant (GRR_{UV6}) was used for NTG treatment[24]. N-Nitroso guanidine mutagenesis was carried out on spores[25] of GRR_{UV6}. The spore suspension was prepared in the same manner as described earlier. To a 9 mL of spore suspension, 1 mL of

sterile solution of NTG (3 mg mL^{-1} in sterile physiological saline) was added. The reaction was allowed to proceed. Samples were withdrawn from the reaction mixture at intervals of 30, 60, 90, 120, 150 and 180 min and immediately centrifuged for 10 min at 5500 rpm and supernatant solution was decanted. Cells were washed three times with physiological saline and resuspended in 10 mL of sterile physiological saline. The samples were serially diluted in the sterile physiological saline and plated over oat meal agar medium as mentioned earlier. A total of 9 colonies (designated as GRR_{NTG1} to GRR_{NTG9}) were selected from the plates showing less than 1 % survival rate (120 and 150 min NTG treated spore suspension) and tested for bioactive metabolites production.

Antimicrobial activity: The antimicrobial activity of the culture filtrate was determined by agar well diffusion method[26]. Wells of 6 mm diameter were prepared in the nutrient agar plates and the test pathogenic bacterial and fungal cultures were swabbed on to the nutrient agar surface[27] and the wells were filled with the 50 µL of crude culture supernatant and the diameter of inhibition zones were measured after incubation for 24 h at 37 ºC for the bacterial species and 48 h at 28 ºC in the case of fungal species. All the experiments were carried out in triplicate and the mean of the three was presented.

RESULTS AND DISCUSSION

In general, strain improvement is considered as one of the main factors involved in the achievement of higher titers of industrial metabolites[28]. Random mutagenesis and fermentation screening have been reported as an effective way to improve the productivity of industrial microbial cultures[1]. The most widely used physical mutagen were ultraviolet (UV) irradiation[29] and chemical mutagens are nitrosoguanidine (NTG), methylmethane sulfonate (MMS), ethylmethane sulfonate (EMS), N-methyl-N-nitrosoguanidine (MNTG).

Mutation was used as a major tool for the induction of a wide range of genetic variations for selection of higher antibiotic producer of *Streptomyces*. After treatment of the wild strain *Streptomyces rochei* with UV irradiation and NTG for different exposure times: 15, 30, 45, 60, 75, 90 min and 30, 60, 90, 120, 150, 180 min, respectively, wild strain were enhanced their productivity of bioactive metabolites was examined.

The bioactive metabolites production by the natural selectants was shown in Table-1, GRR_{NS3} showed highest bioactive metabolites production, against pathogenic test microorganisms were *Candida albicana* (MTCC 183), *Staphylococcus aureus* (MTCC 3160) and *Escherichia coli* (MTCC 443), was measured by inhibition zone diameter were 20, 20 and 17 mm (111, 105 and 113 %), respectively, which was averagely 110 % higher than the wild strain. Also, only one other natural selectants GRR_{NS7}, showed higher antimicrobial activity than the wild strain. The natural selectants GRR_{NS3} was choosen for further strain improvement employing UV irradiation.

It was suggested that 99.9 % kill is best suited for strain improvement as the fewer survivor in the treated sample will have undergone repeated or multiple mutations which may lead to the enhancement in the productivity of the culture[25]. In the present study, the plates having less than 1% survival rate (45, 60 and 75 min) were selected for the isolation of UV mutants. A total of 9 mutants were selected and tested for antimicrobial metabolites production was shown in Table-2. Three UV mutants (GRR_{UV1}, GRR_{UV6} and GRR_{UV7}) showed higher antimicrobial activity than the parent strain (GRR_{NS3}). In the present study, the high yield antimicrobial metabolites produced UV mutant GRR_{UV6} was measured by inhibition zone diameter were 25, 23 and 20 mm (120, 115 and 118 %), respectively, which was averagely 118 % higher than the parent strain (GRR_{NS3}) and 129 % higher than the wild strain.

TABLE-1
MODIFIED ANTIMICROBIAL SPECTRA OF NATURAL SELECTANTS AND THEIR ZONE OF INHIBITION

Natural selectants	Inhibition zone diameter (mm)		
	Pathogenic test microorganisms		
	C. albicana (MTTC 183)	*S. aureus* (MTTC 3160)	*E. coli* (MTTC 443)
GRR_{NS1}	16	17	12
GRR_{NS2}	14	15	11
GRR_{NS3}	20	20	17
GRR_{NS4}	17	18	13
GRR_{NS5}	12	14	10
GRR_{NS6}	10	11	9
GRR_{NS7}	19	18	15
GRR_{NS8}	Nil	Nil	Nil
GRR_{NS9}	13	14	11
Wild strain	18	19	15

TABLE-2
MODIFIED ANTIMICROBIAL SPECTRA OF UV MUTANTS AND THEIR ZONE OF INHIBITION

UV mutants	Inhibition zone diameter (mm)		
	Pathogenic test microorganisms		
	C. albicana (MTTC 183)	*S. aureus* (MTTC 3160)	*E. coli* (MTTC 443)
GRR_{UV1}	22	21	18
GRR_{UV2}	10	12	08
GRR_{UV3}	Nil	Nil	Nil
GRR_{UV4}	11	12	09
GRR_{UV5}	15	18	14
GRR_{UV6}	24	23	20
GRR_{UV7}	23	22	15
GRR_{UV8}	18	19	16
GRR_{UV9}	Nil	Nil	Nil
Parent strain (GRR_{NS3})	20	20	17

N-Nitroso guanidine is also considered to be a very effective mutagen[30,31] since this compound is known to induce multiple, clustered lesions and has been shown to be ideal for isolating mutants with variations in antibiotic yields by *Streptomyces fradiae*[29] and by *Streptomyces coelicolor*[32]. In the present study, the UV mutant GRR_{UV6} was selected and was next subjected to further strain improvement by NTG treatment for different time intervals (30, 60, 90, 120, 150 and 180 min). Plates having less than 1 % survival rates (120 and150 min) were selected for the isolation of mutants and the antimicrobial activity of the selected NTG mutants is presented in Table-3. GRR_{NTG8} antimicrobial activity was measured by inhibition zone diameter were 26, 25 and 23 mm (108, 109 and 115 %), respectively, which was averagely 111 % higher than the parent strain

TABLE-3
MODIFIED ANTIMICROBIAL SPECTRA OF NTG MUTANTS AND THEIR ZONE OF INHIBITION

NTG mutants	Inhibition zone diameter (mm)		
	Pathogenic test microorganisms		
	C. albicana (MTTC 183)	*S. aureus* (MTTC 3160)	*E. coli* (MTTC 443)
GRR_{NTG1}	10	12	09
GRR_{NTG2}	18	18	15
GRR_{NTG3}	17	18	14
GRR_{NTG4}	Nil	Nil	Nil
GRR_{NTG5}	19	20	16
GRR_{NTG6}	25	23	21
GRR_{NTG7}	15	14	11
GRR_{NTG8}	26	25	23
GRR_{NTG9}	20	19	14
Parent strain (GRR_{UV6})	24	23	20

(GRR_{UV6}) and 143 % higher than the wild strain. Hence, present study of *the Streptomyces rochei* (Isolate No. 10) showed productivity up to 111-143 %. Whereas, the productivity of *S. hygroscopicus* FC904 (the producer of rapamycin) up to 60-124 % only[33], after mutagenesis by UV and NTG.

The literature reported that, the search for bioactive components with potential medical application, the marine *Streptomyces* species isolate AH2 was subjected to mutation UV-light and selected superior mutants compared with the wild type strain for their bioactive metabolite production[23] and the prolific producer of bioactive UV mutant of *Streptomyces* 10/14, up scaled and the hyper produced bioactive constituents isolated[18]. And also, *S. olivaceus* subjected in UV irradiation leading to mutations different from the original strain and showed variation in their spectrum of antimicrobial activity[34]. Production of high titre of tylosin by hyper-producing mutants after treatment of *S. fradiae* NRRL2702 was observed[35] by treating with N-nitrosoguanidine (NTG) or exposing to UV.

Conclusion

Improvement of microbial strains for the over-production of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of the process with increased productivity and may also possess some specialized desirable characteristics. Effectiveness of UV radiation (physical mutagen) and NTG (chemical mutagen) in strain improvement for enhanced antibiotic metabolites productivity was demonstrated in the present investigation. In the present study, a mutant, *Streptomyces rochei* GRR368 (Isolate No.10) capable of showing hyper-productivity of antibiotic metabolites was isolated using successive mutation procedures UV-irradiation followed by NTG treatment of the spores. From the above study, the mutations of the marine *S. rochei* by UV treatment and NTG affected the production of bioactive metabolites quantitatively. It is hoped that the high yielding mutant strain of the isolate *Streptomyces rochei* GRR368 (Isolate No.10) can be exploited commercially for large scale industrial production of bioactive metabolites.

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