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# MUTATION OF *CEPHALOSPORIUM ACREMONIUM* NRRL-2443 THROUGH UV-IRRADIATION FOR CEPHALOSPORIN-C PRODUCTION

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## ABSTRACT

The present study is concerned with the selection of a mutant variant of *Cephalosporium acremonium* for the optimal production of cephalosporin-C. The parental strain *C. acremonium* NRRL-2443 was subjected to UV-radiations for 10-80 min. Among all the mutants tested, *C. acremonium* $GC_{UV-6}$ gave maximum antibiotic i.e. 454 mg/ml, which was 3.91 fold improved compared to the wild (116 mg/ml). The cultural conditions and nutritional requirements for cephalosporin-C production by selected mutant were optimized by submerged culture technique. The incubation period (72 h), initial pH (6.5) and soybean meal (6.0 %, w/v) supported maximum production of cephalosporin-C (493 mg/ml).

## INTRODUCTION

Cephalosporins are semi synthetic antibiotic derivatives of cephalosporin-C, produced mainly as secondary metabolites. Many microbial strains including *Cephalosporium acremonium, C. salmosynnenstum, Streptomyces clavuligenous, S. lactamgenes and Acremonium chrysogenum* are known to produce cephalosporins, however, *C. acremonium* is a widely used strain in biotechnology because of its high antibiotic activity (Brana *et al.,* 1985, Srivasta *et al.,* 1996). The production of cephalosporins can be increased by strain improvement based on random mutation and screening. Mutation can enhance the efficiency of some genes through duplication or depletion mechanisms (Stadler, 1997, Ellaiah *et al.,* 2002). Antibiotic production could also be increased by the nutrient controlled metabolism and also by optimizing the conditions necessary for fermentation. The optimal time of incubation for cephalosporin-C fermentation varies with the fermentation conditions. Maintenance of a favorable initial pH is one of the most important steps for the successful progression and termination of fermentation (Metsumara *et al.,* 1978, Herold *et al.,* 1988, Araujo et *al*., 1999). The type and concentration of nitrogen source in the medium affects both mycelial growth and cephalosporin-C production. The medium containing soybean meal as an organic nitrogen source can give maximum production of cephalosporin-C (Shen *et al.,* 1986).

Large quantities of by-products and raw materials such as molasses are being produced which are available for their exploitation as substrate, hence can also be used for the biosynthesis of cephalosporin-C. The present study is concerned with the screening of UV mutated variants of *C. acremonium* and optimization of cultural conditions for enhanced production of cephalosporin-C in batch culture.

## MATERIALS AND METHODS

Organism: Cephalosporin-C producing strain (*Cephalosporium acremonium* NRRL-2443) was obtained from IIB, GCU

Lahore. The culture was maintained on the nutrient agar medium containing (%, w/v): 2.0 sucrose, 0.05 $K_2HPO_4$, 0.05 $KH_2PO_4$, 0.05 KCI, 0.05 $MgSO_4.7H_2O$, 0.001 $FeSO_4$ $.7H_20$, 0.3 $NaNO_3$, 0.4 yeast extract, 0.4 peptone, 2.0 agar, initial pH 6.0.

All the chemicals, unless otherwise stated, were of analytical grade and obtained directly from Sigma (USA), BDH (UK), E-Merck (Germany), Acros (Belgium) or Fluka (Switzerland). Strain improvement through UV-radiations: The culture of *C. acremonium* NRRL-2443 was improved by UV-radiations for the production of cephalosporin-C. Ten millilitre of sterilized distilled water was added to a 3-4 day old slant culture. An inoculum needle was gently used to prepare spore suspension. One millilitre of the suspension was aseptically transferred to 100 ml of nutrient medium. The flask was placed in a rotary shaking incubator (Model: GLSC 051.HR.196-11, Pak made) at 160 rpm (30°C) for 24 h. One millilitre of the inoculum was diluted up to $10^4$ times with distilled water. Five millilitre of the diluted suspension was exposed to UV-radiations for different time intervals (10-80 min) under a UV lamp (Mineral Light UVS.12, California, USA, λ=253 nm at 50 cycles/S, 220 V). Dose given to the suspension was 1.2 x $10^2$ J/$m^2$/S. The distance between lamp and suspension was adjusted to 6 cm for each trial. After each interval about 0.5 ml the suspension was transferred to individual plates containing 2.0 % (w/v) oxgal nutrient agar medium. The plates were incubated at 30°C for 72 h to obtain maximum growth. The mutant variants were selected from the plates having at least 90 % death rate after Das and Nandi (1969). The selected mutants were transferred to nutrient agar medium slants and incubated at 30°C for 72 hours. The cultures were stored at 4°C in a refrigerator.

**Molasses pre-treatment:** Cane molasses obtained from Kamalia sugar mills (Kamalia, Pakistan) was used in the present study. Sugar content of molasses was about 45 % (w/v). It was boiled for half an hour followed by the addition of 35 g/l 1.0 N $H_2SO_4$ and allowed to stand overnight. The clear supernatant was diluted to desired sugar level.

**Batch culture methodologies:** One millilitre of the spore suspension (1.45 × $10^6$ /ml) was aseptically transferred to a flask containing 100 ml of sterilized nutrient broth medium with (% w/v): 0.5 peptone, 0.5 beef extract, 0.3 NaCl, initial pH 6.0. The flask was placed in a rotary shaking incubator (200 rpm) at 30˚C for 24 h. Twenty-five millilitre of the fermentation medium containing (%, w/v): 2.0 molasses sugar, 0.8 ammonium acetate, 3.0 corn starch, 5.0 sugarcane molasses, 6.0 soybean meal, 0.5 $CaCO_3$, 1.25 $CaSO_4$, 3.0 methyl oleate, initial pH 6.5 was transferred to individual flasks. One millilitre of the inoculum was aseptically transferred to each flask. Flasks were incubated in a rotary shaking incubator at 30°C for 72 h (200 rpm). After 72 h, the fermented broth was centrifuged at 6,000 rpm for 20 min and supernatant was used for the estimation of cephalosporin-C. All the experiments were run parallel in triplicates.

**Assay methods:** Three millilitre of the supernatant was transferred to test tubes and 2.0 ml of nickel reagent was added into it. The tubes were placed at 25°C for 20 min. Then 5.0 ml of iron reagent was added to each tube and allowed to react at 30˚C for 2 h. Optical density was measured at 470 nm with a spectrophotometer and converted into mg/ml from the standard curve of cephalosporin-C (Mays *et al.,* 1975). Significance difference among the replicates has been presented as Duncan's multiple ranges in the form of probability

(<p>) values (Snedecor and Cochran, 1980).

## RESULTS AND DISCUSSION

The parent culture of *Cephalosporium acremonium* NRRL-2443 was subjected to UV-radiations for 10-80 min. Out of 32 mutant variants selected after preliminary selection, 8 were tested for cephalosporin-C production by batch culture (Table 1). *C. acremonium* $GC_{UV-6}$ (subjected to UV-radiations for 40 min) gave maximum production (454 mg/ml), which was 3.91 fold improved compared to the wild (116 mg/ml). The selection and formulation of a suitable medium is necessary for the growth of fungi as well as bacteria for the production of antibiotics (Kim *et al.,* 2003).

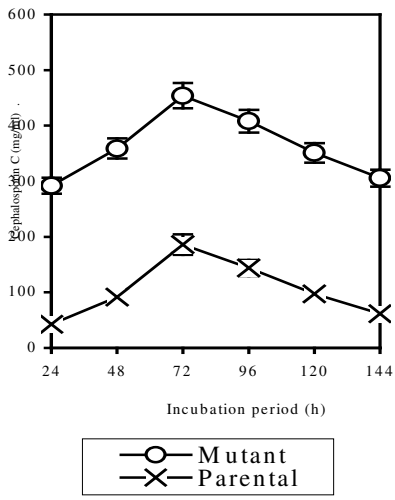
**Table-1:** Screening of UV-irradiated variants of *C. acremonium* for cephalosporin-C production.

| Mutant variants of *C. acremonium* | Cephalosporin-C (mg/ml) |
|---|---|
| Parental (NRRL-2443) | $116\pm2.1^{hij}$ |
| $GC_{UV-1}$ | $277\pm3.0^{e}$ |
| $GC_{UV-2}$ | $289\pm5.1^{de}$ |
| $GC_{UV-3}$ | $304\pm3.0^{d}$ |
| $GC_{UV-4}$ | $311\pm2.5^{d}$ |
| $GC_{UV-5}$ | $367\pm2.5^{bc}$ |
| $GC_{UV-6}$ | $454\pm2.5^{a}$ |
| $GC_{UV-7}$ | $293\pm3.6^{de}$ |
| $GC_{UV-8}$ | $258\pm2.5^{ef}$ |

Incubation period 72 hours, initial pH 5.5, temperature 30°C. ± Indicate standard deviation among the three parallel replicates. The values designated by letters differ significantly at $p\leq0.05$.

The cultural conditions optimized during the study were incubation time, initial pH and nitrogen source. The cephalosporin-C fermentation was carried out for 24-144 hours at 30ºC (Fig. - 1). The production of antibiotic increased with the increase in time and found to be optimal (493mg/ml) after 72 h inoculation and these are substantiating with the observations of Trown *et al.,* (1962). However, Metsumara *et al.,* (1978) optimized incubation time of 96-120 hours for maximum production of cephalosporin-C. As the incubation time increased beyond the optimal, antibiotic activity decreased gradually. It might be due to the decreased amount of available nutrients, age of fungi and accumulation of by-products in the medium (Seidel *et al.,* 2000).

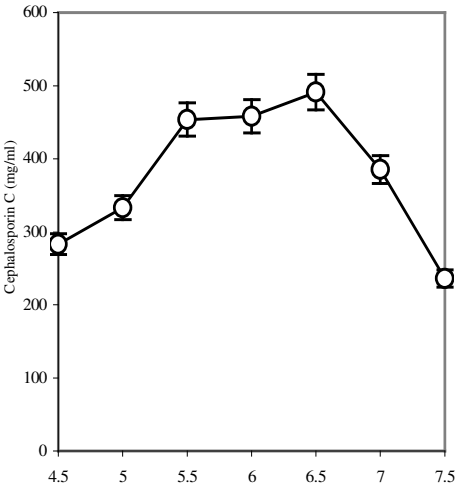
**Fig.-1:** Rate of cephalosporin-C production by *C. acremonium* $GC_{UV-6}$




Initial pH 5.5, temperature 30°C. Y error bars indicate the standard deviation among the three parallel replicates.

The effect of initial pH (4.5-7.5) on the production of cephalosporin-C was also studied (Fig.- 2). Maximum antibiotic production (491mg/ml) was obtained when pH of fermentation medium was adjusted to 6.5. The range of initial pH (6.5-7.7) for cephalosporin-C biosynthesis has reported by previous workers (Trown *et al*., 1962, Drew and Demain, 1975, Shen *et al*., 1986). At a lower pH the antibiotic activity remained low. It might be due to the fact that production of biomass was greatly decreased due to the toxic effect of $H^+$ ions. Zhou *et al*., (1992) have reported similar kinds of findings. At a higher pH (beyond the optimal), the productivity was inhibited might be due to improper or disturbed growth of mycelium.

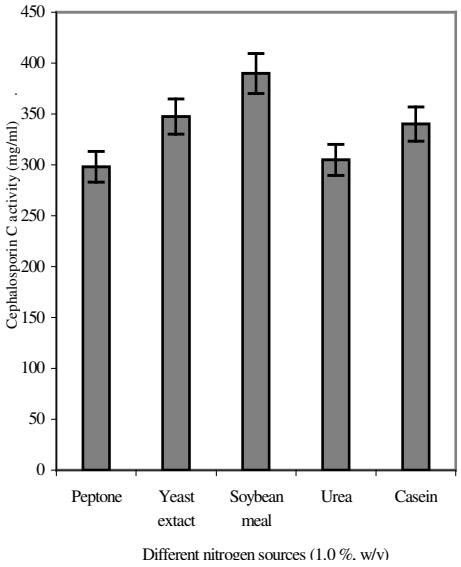
**Fig.-2:** Effect of initial pH on cephalosporin-C production by *C. acremonium* $GC_{UV-6}$.




Incubation period 72 h, temperature 30°C. Y error bars indicate the standard deviation among the three parallel replicates.

Different nitrogen sources such as peptone, yeast extract, soybean meal, urea and casein were added (at 1.0 %, w/v level) in the fermentation medium individually to check their antibiotic potential (Fig.-3). Among these, soybean meal was optimized as the best nitrogen source for maximum production of cephalosporin-C.

**Fig**.- 3: Effect of different nitrogen sources on cephalosporin-C production by *C. acremonium* $GC_{UV-6}$.

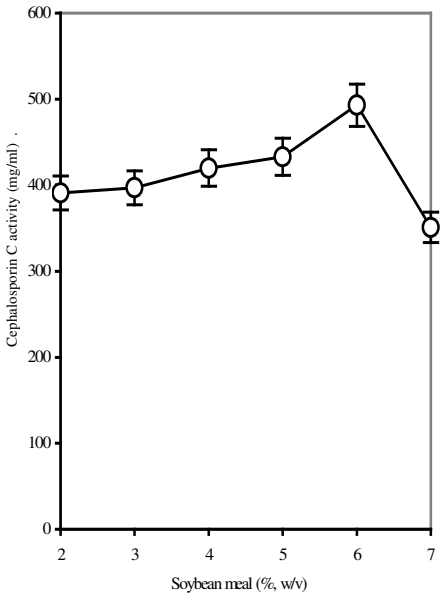



Incubation period 72 h, initial pH 6.5, temperature 30°C. Y error bars indicate the standard deviation among the three parallel replicates.

Optimal concentration of soybean meal was determined by varying its concentration from 2.0-7.0 % (w/v). Antibiotic activity was found to be optimal (493 mg/ml) at 6.0 % (w/v) level (Fig. -4). At a lower concentration, less antibiotic production might be due to the lower supply of available nitrogen for mycelial growth. On the other hand, at larger nitrogen quantity the biomass grew slower and hence, cephalosporin-C production decreased gradually. Similar kinds of

findings have also been reported earlier (Shen *et al*., 1986).

Fig.-4: Effect of different concentration of soybean meal cephalosporin-C production by *C. acremonium* $GC_{UV-6}$.




Incubation period 72 h, initial pH 6.5, temperature 30°C. Y error bars indicate the standard deviation among the three parallel replicates

## CONCLUSION

*C. acremonium* NRRL-2443 was mutated through UV-irradiation to enhance its antibiotic potential in batch culture. Among the 32 mutant variants, $GC_{UV}$-6 gave 3.91 fold improved cephalosporin-C production compared to the wild strain. The cultural conditions viz. incubation period (72 h), initial pH (6.5) and soybean meal (6.0 %, w/v) were also optimized. The maximum cephalosporin-C production achieved during the course of study was 493 mg/ml, which is highly significant ($p \leq 0.05$).

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