PRODUCTION OF ACTINOMYCIN-D BY THE MUTANT OF A NEW ISOLATE OF *STREPTOMYCES SINDENENSIS*

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ABSTRACT

An actinomycin-D producing strain was isolated from soil and characterized as *Streptomyces sindenensis*. The culture was subjected to UV irradiation and a mutant with 400% higher actinomycin-D production was isolated (400 mg/l^{-1} as compared to 80 mg/l^{-1} produced by the parent). Production medium was optimized and antibiotic yield with the mutant was enhanced to 850 mg/l^{-1} which is 963% higher as compared with the parent.

Key words: Actinomycin-D, antibiotics, *Streptomyces*, mutation

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The actinomycins are chromopeptide lactone antibiotics, of which more than 30 natives are known. Among the actinomycins, actinomycin-D (act-D) has been studied extensively and is used clinically for the treatment of Wilms' tumor (3). Various strains of *Streptomyces* and *Micromonospora* are reported to produce different forms of actinomycins (9). Production and optimization of act-D by *S. sindenensis* is not reported in literature. Present study reports isolation of a high yielding mutant of *S. sindenensis* for the production of act-D. Bioprocess parameters for the mutant cultivation and optimum product formation were studied.

The producer microorganism, designated as C-5, was isolated from the soil sample, collected from steel plant effluents (Barabanki, U.P., India) and maintained on ISP-2 (International *Streptomyces* Project) agar slants containing glucose 4g, yeast extract 4g, malt extract 10g, CaCO$_3$ 2g, agar 20g and distilled water 1L, pH adjusted to 7-7.2 before sterilization. Morphological and cultural characteristics were studied using the ISP media recommended by Shirling and Gottlieb, 1966 (11). For Scanning Electron Microscopy (SEM) samples were prepared according to the methods described by Castillo *et al.* (2). The strain was characterized as *S. sindenensis* by 16S rRNA homology (data not shown) and has been deposited at MTCC (www.http:// mtcc.imtech.res.in), Chandigarh, India (MTCC 8122). Nearly complete (1366 bp) 16S rRNA sequence of strain has been submitted in the NCBI Gen Bank database (accession number EF422787).

Antibiotic production was studied in shake flask with the production medium containing: soy bean meal 10 g, glycerol 15 ml, MgSO$_4$.7H$_2$O 0.5 g, (NH$_4$)$_2$HPO$_4$ 0.5 g, K$_2$HPO$_4$ 1.0 g, NaCl 3 g, CaCO$_3$ 2g, and distilled water 1L, pH adjusted to 7-7.2 before sterilization in autoclave at 15 lb PS i (1 Kg PS cm) for 15 minutes. Seed culture was prepared in 250 ml Erlenmeyer flask containing 50 ml of production medium by inoculating a loop full culture from the slant and incubating at 28ºC on rotary shakers at 200 rpm for 48 h. Antibiotic production was observed in the same medium by inoculating 1L flask (200 ml medium) with 2.5% (v/v) of seed culture and growing under the same conditions for 168 h. Fermented broth was centrifuged and supernatant was extracted thrice with ethyl acetate, filtered and concentrated invaccuo. Antibiotic titer was estimated by the Reverse phase HPLC with ODS-3 column (outer dia.., 250 x 6.35 mm) with a particle size of 10 µ and Lambda-Max Spectrophotometer- LC 481 variable wavelength detector 254 nm, at a flow rate of 0.6 ml/min. with acetonirile:water (55:45) as eluting solvent. Act-D was eluted at 21.4 min retention time. Unless otherwise stated, all the chemicals and media components were purchased from Hi-media labs, Mumbai, India.

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Bacillus subtilis ATCC 6633 was used as the test strain for the bioassay of antibiotic production.

Spores of *S. sindenensis* were gently scrapped from the surface of ISP-2 agar plates, washed with sterile normal saline (0.85%) and filtered through glass wool. Spore suspension was diluted to have a count of 10^4/ ml as, determined by the viability observed on ISP-2 agar plate. Three ml of spore suspension was irradiated for 30 min. with a UV lamp (254 nm) placed about 30 cm above from the liquid surface and gently swirled (magnetically with a needle) in a Petri dish (covered by a piece of dialyzing membrane). Following irradiation, spores were kept in dark at 4ºC overnight.

Rifampicin (Rf) and streptomycin (S) were used as screening agents for identification of mutants among the survivors. After incubation in dark, spores were plated in triplicates on ISP-2 agar plates containing 25 µg ml^{-1} of S and Rf each, incubated at 28ºC and observed after 48 hr. Mutant colonies with different morphology and expressing, successively, resistance to S and Rf were isolated. Antibiotic production by mutants was studied with 10 µg ml^{-1} of S and Rf added in shake flasks. Parallel control flasks were also run for the parent culture. Confirmation for improved antibiotic production was done in shake flasks with no addition of S and Rf.

Effects of different carbon and nitrogen sources on antibiotic production were evaluated for medium optimization (Table 1). Glycerol was replaced with different sugars (1%). Amino acids were supplemented to complete production medium (0.1%). Mutant was cultivated in optimized medium using NBS BioFlow 110 bench top stirred bioreactor.

The rate of survival of UV irradiated cell progenies was 1%. Fifty colonies, showing resistance for higher concentration of Rf and S (25 µg ml^{-1} each) and higher zone of inhibition against *B. subtilis* were selected for further studies. One such mutant designated as M-46, produced 5 folds higher act-D (400 mg/l^{-1}) as compared to the parent strain (80 mg/l^{-1}).

The parent and M-46 were studied for their cultural characteristics with respect to the utilization of nitrate, urea and sodium citrate. M-46 utilized Sodium citrate while the parent did not. M-46 better utilized other substrates (starch, asparagine, yeast extract and tyrosine) as evidenced by the colony size and pigment production. Growth of parent and M-46 was also observed on medium ISP 2 - 7 media. The parent strain did not produce melanin on ISP-6 agar where as M-46 produced brown color indicating the production of melanin. On ISP-2 agar plates, M-46 showed vigorous sporulation and higher pigment production. As shown in SEM images (Fig. 1) M-46 spores were stouter (0.7×0.5 µm) as compared to spores produced by the parent strain (0.7×0.3 µm).

Influence of different carbon and nitrogen sources on act-D production by parent strain and M-46 is given in Table 1. Most of the medium components that favoured act-D production by the parent strain also favoured mutant. It is evidenced from Fig. 1 (C & D) that act-D production by the mutant was enhanced.

Negative effect exerted by glutamate and aspartic acid (29 and 45% decreased production) on antibiotic production by the mutant was the only contradiction observed. Replacement of glycerol with fructose gave an enhancement of 65 and 138% in the antibiotic titer of M-46 and parent strain respectively (Table 1). Incorporation of DL-threonine in the production medium also favoured act-D production by the mutant as well as parent strain (55 and 57% respectively). Finally a production medium containing: soy bean meal 10 g, fructose 15 g, DL-threonine 1 g, $MgSO_4.7H_2O$ 0.5 g, $(NH_4)_2HPO_4$ 0.5 g, K_2HPO_4 1.0 g, NaCl 3 g, $CaCO_3$ 2g, and distilled water 1L, pH adjusted to 7-7.2, was used for act-D production by M-46 giving an enhancement of 77% (710 mg/l^{-1}) as compared to the yield obtained with M-46 in normal production medium. When M-46 was cultivated in stirred bioreactor with aeration and agitation rates of 1.5 vvm and 600 rpm respectively, productivity was enhanced by 963% (850 mg/l^{-1}) as compared to the parent strain, cultivated in shake flasks with non-optimized medium (80 mg/l^{-1}).

In our study, we have observed an enhancement in act-D synthesis by the introduction of Rifampicin and Streptomycin resistance into *S. sindenensis*. Introduction of certain mutations into *rpo gene* that confer resistance to Rifampicin are reported to activate the antibiotic production by *Streptomyces* spp. (5).

Table 1. Influence of C & N sources on act-D production by parent and mutant.

| C & N sources	dry cell weight, g l^{-1}		act-D, mg l^{-1}	
	parent	M-46	parent	M-46
replacement of glycerol with (1%)				
fructose	7.2	6.2	190	660
lactose	6.8	6.0	156	510
maltose	7.0	6.0	130	590
mannose	7.5	7.0	70	369
xylose	7.5	6.2	110	480
production medium	7.0	6.1	80	400
supplimentation of production medium with (0.1%)				
L-asparagine monohydrate	6.8	6.0	66	440
DL-aspartic acid	7.5	6.2	148	220
L-glutmate	7.0	5.5	141	284
L-histidine	6.8	6.0	110	335
Hydroxy L-proline	6.9	6.5	90	330
DL-isoleucine	6.8	7.2	88	286
DL-serine	6.8	6.0	64	266
DL-threonine	7.2	6.5	126	620
L-tryptophan	7.5	6.2	74	438
Optimized medium	6.8	6.2	215	710
Optimized medium (bioreactor)	7.0	6.8	270	850
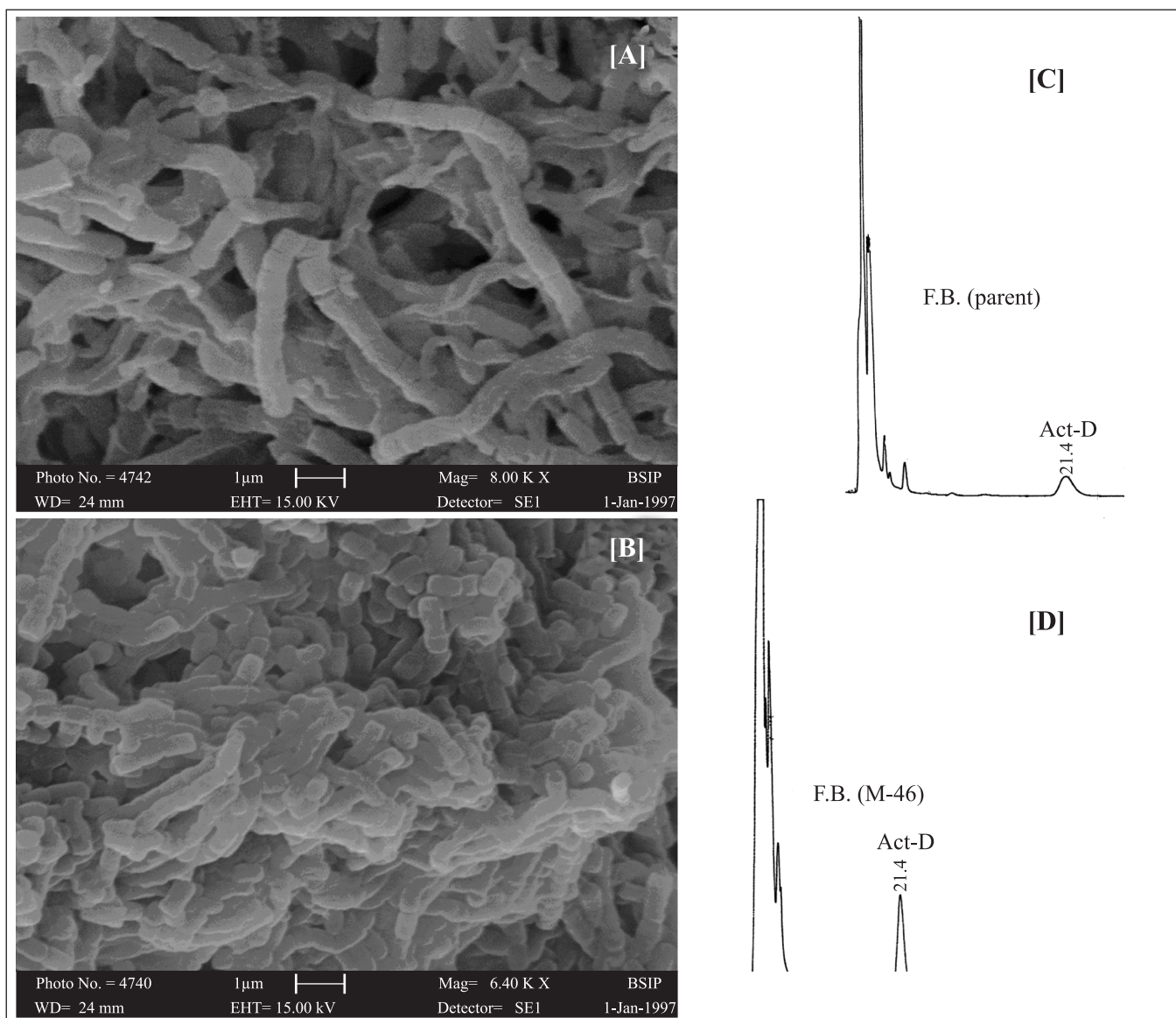
Figure 1. A & B, showing SEM images of parent and mutant strain and C & D showing the HPLC profile of fermented broth (F.B.) of parent and mutant strains respectively.

DNA-dependent RNA polymerase (RNAP), which is composed of an essential catalytic core enzyme ($\alpha_2\,\beta\,\beta\,\omega$) and one of the sigma ($\sigma$) factors, is the central enzyme for the expression of genomic information in all organisms. Rifampicin (*Rif*) inhibits transcription initiation by blocking the subunit of bacterial RNA Polymerase (7). Resistance to streptomycin is brought about by mutation in the *rpsL* gene which encodes for the S12 protein of the 30S subunit of the ribosome (10).

In our studies, fructose was found to induce a substantial increase in act-D production by the mutant and parent both. Inbar and Lipidot (6) showed that carbon atoms of an intracellular glutamate pool of *S. parvullus* were not derived biosynthetically from the culture medium glutamate source but rather from fructose catabolism. Foster and Katz (4) found that in case of *S. parvullus* use of L-glutamate and L-aspartate as a C-source exerts catabolic repression on synthesis of tryptophan oxygenase, an enzyme needed for the synthesis of actinomycin. Perhaps in case of M-46 this catabolic repression has got expressed at a higher level as L-glutamate and DL-aspartic acid supplementations were found to inhibit the production of act-D significantly (29 and 45% respectively). Some nitrogen sources may get incorporated in antibiotic molecules as

precursors or their amino groups transfer to specific intermediate products (1). Katz and Goss (8) have reported up to 83% enhancement in act-D production by *S. chrysomallus* with the addition of DL-valine in the production medium. In our studies, positive effects of DL-threonine, L-valine and proline could be due to their direct incorporation in the peptide chains attached to the chromophore (actinocin) of act-D molecule.

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RESUMO

Produção de actinomicina-D por um mutante de uma nova cepa de *Streptomyces sindenensis*

Uma cepa produtora de actinomicina-D foi isolada de solo e caracterizada como *Streptomyces sindenensis*. A cultura foi submetida à radiação UV, e um mutante capaz de produzir 400% mais actinomicina-D foi isolado (400mg/L comparado a 80mg/L produzido pela cepa parental). O meio de produção do antibiótico foi otimizado e o rendimento aumentou para 850 mg/L, ou seja, 963% mais alto que a cepa parental.

Palavras-chave: actinomicina-D, antibióticos, *Streptomyces,* mutação

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