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Short communication

Enhancement of gentamicin production by mutagenesis and non-nutritional stress conditions in *Micromonospora echinospora*

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Abstract

Gentamicin producing strain of *Micromonospora echinospora* was treated with chemical mutagens like EtBr and MNNG and physical mutagens such as UV was carried out to obtain a mutant with enhanced production of gentamicin. After inducing mutations screening for penicillin and kanamicin resistant mutants was done. *M. echinospora* EtBr-22 strain was obtained by mutations and its gentamicin production in shake flask reaches 1354 mg l^{-1} which is 1.53-fold higher than that of the parent strain. Application of different stress conditions like heat shock, feeding high ethanol and high NaCl concentrations during fermentation has found to be effective for the increased production of gentamicin. Production of gentamicin was increased to 1.26-fold in medium supplemented with 0.6% NaCl to 48-h-old culture.

Keywords: *Micromonospora*; Gentamicin; Mutations; Stress conditions; Heat shock; Salt stress

1. Introduction

Fermentation by *Micromonospora* species, *echinospora* and *purpurea* produces a family of aminocyclitol antibiotic called gentamicin [1]. Gentamicin is a basic, water soluble antibiotic with a wide spectrum of antimicrobial activity and is still considered cornerstone in the treatment of several infections caused by gram-negative aerobic bacteria, first reported by Weinstein et al. [2].

The industrial potential of antibiotics has stimulated research in the development of methods to improve strains, since the production level of antibiotics in naturally occurring strains is sometimes low for commercial exploitation. For the development of a commercially feasible fermentation process, improvement in yield and overall productivity are essential. Strain improvement by induced mutagenesis has been developed with rational selection procedures for an efficient screening of the enhanced antibiotic producing mutants [3,4]. Although appreciable enhancement of gentamicin titer in laboratory experiments has been published [5,6], no significant improvement in the commercial production has been reported. So the mechanisms of antibiotic production, strain improvement for the productivity are important themes.

A stress-mediated bioprocess was another designed strategy to enhance biological target productivity [7]. Among stressors, heat treatment was a sensitive and useful stimulus for biological cells such as bacteria. In nature, the production of secondary metabolites such as antibiotics by microorganisms increases as a response to biotic and abiotic stress. Temperature [8], NaCl [9] ethanol [10] and pH [11] were an important non-nutritional stress factors influencing the secondary metabolites production.

In this study, we treated *Micromonospora echinospora* with different mutagens ultra violet (UV), ethidium bromide (EtBr) and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) for a hyper gentamicin producing strain. Also investigated the effect of different non-nutritional stress methods imposed by heat shock in water bath and microwave oven, high NaCl concentration and high ethanol during the course of fermentation and the stress conditions were optimized.

2. Materials and methods

The parent strain *M. echinospora* subsp. *pallida* (ATCC 15838, MTCC 708) procured from MTCC, Institute of Microbial Technology, Chandigarh, India was used for mutation and stress methods described in this study. Inoculation medium contained (g l^{-1}): beef extract, 3; glucose, 1; soluble starch, 24; yeast extract, 5; $CaCO_3$, 4; and pH 7.6. Each 250 ml Erlenmeyer flasks contained 50 ml inoculum medium and inoculated with *M. echinospora* (one slant for each

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flask) under aseptic conditions. The inoculated flasks were kept on a rotary shaker at 200 rpm at 27 ± 2 °C for 72 h. A 3 ml sample of 72-h-old *M. echinospora* culture was added to a 250 ml Erlenmeyer flask containing 50 ml of fermentation medium contained (g l^{-1}): starch, 9; soyabean meal, 3; K_2HPO_4, 0.9; $CaCO_3$, 4; $FeSO_4$, 0.03; $CoCl_2$, 0.001 and cultivated for 7 days at 28 °C [12].

2.1. Mutations

A 10 ml of mycelial suspension from a slant culture of *M. echinospora* (approximately 2×10^7 cells ml^{-1}) was treated with 200 and 500 μg ml^{-1} of EtBr and MNGG, respectively for duration of 30 min and 1 h. These concentrations were optimized to get the survival frequency of 0.04% (data not present). A 10 ml of mycelial suspension taken in the aseptic plate without cover was exposed to UV light for 10, 20 and 30 min at a distance of 30 cm from UV lamp with a wave length of 2537 Å and a power of 30 W to get a survival frequency of 0.01–0.1%.

Penicillin (50 μg ml^{-1}) and kanamicin (50 μg ml^{-1}) were used as screening agents to obtain drug-resistant mutants. The cell suspension of the parent strain after inducing mutations was spread on the medium containing one of the above agents and the resistant mutants were screened. A total of 75 colonies were isolated and after shake flask fermentations gentamicin production was measured and compared.

2.2. Stress methods

M. echinospora was cultivated in the production medium under stress conditions like heat shock, NaCl and ethanol. For heat shock treatment the cultures were exposed to different temperatures of 40, 45 and 50 °C for 1 min in a hot water batch and then back to 28 °C. Since the optimal gentamicin production was observed at 45 °C further experiments were carried out at this temperature. Further, during the fermentation at every 24 h interval (up to 72 h) the cultures were exposed to heat shock at 45 °C for 1 min and the gentamicin production was estimated.

Microwave oven (Model BPL, BMD800 IS, India) with a capacity of 800 W of microwave output power at 2460 MHz used for heat shock treatment with allowable output of 150 W. Constant power output (150 W) was used throughout the study. Production media in three flasks were exposed to microwave oven at different time periods such as 30, 40 and 50 s and the corresponding temperatures recorded in flasks were 40, 45 and 50 ± 2 °C, respectively. Four 250 ml flasks contain 50 ml production media was taken and heat shock was given to these flasks at different incubation periods of 0, 24, 48 and 72 h for 40 s (45 °C ± 2 in a microwave).

Five levels of sodium chloride (0.2, 0.4, 0.6, 0.8 and 1%) were added to 50 ml of production medium on 0, 24, 48, or 72 h of incubation period and gentamicin production and growth of *M. echinospora* was recorded.

To test the ethanol stress, cultures were supplemented with different concentrations of ethanol (1, 2, 3, 4, 5 and 6%) on 0, 24, 48, or 72 h of incubation period and gentamicin production and growth of *M. echinospora* was recorded.

2.3. Analytical methods

At specified intervals, production of gentamicin was determined by an agar disc technique using *Staphylococcus aureus* MTCC 737 as the assay organism [13]. After cultivation, growth was measured as the weight of cell mass obtained from a culture by vacuum filtration (Whatman No.1 filter disc) and drying at 100 °C until constant weight (dry weight basis). All experiments were performed in triplicates, and analyses were carried out in duplicates. The data given here are the means of the measurements.

3. Results and discussion

3.1. Gentamicin production by mutants resistant to penicillin or kanamycin

Mutants isolated on plates having penicillin showed good production than kanamicin resistant mutants. Chu et al. [14] observed that enhancement in the gentamicin titer was due to promotion of secretion of the product from cell wall by

Table 1
Comparison of gentamicin production in UV, EtBr and MNNG mutants

Sl. no.	Mutant name	Time of exposure	Antibiotic used for selection	Gentamicin productivity (mg l^{-1})[a]	Biomass (g l^{-1})[a]
1	UV-3	10 min	Penicillin	987 ± 6.55 (113)	7.9 ± 0.17
2	UV-6	20 min	Penicillin	1146 ± 3.60 (130)	7.2 ± 0.26
3	UV-9	20 min	Kanamicin	1025 ± 8.50 (116)	7.5 ± 0.20
4	UV-14	20 min	Penicillin	1048 ± 4.96 (119)	6.9 ± 0.16
5	UV-17	20 min	Penicillin	1269 ± 5.13 (144)	8.0 ± 0.19
6	UV-20	30 min	Kanamicin	1114 ± 7.46 (127)	7.6 ± 0.22
7	UV-21	30 min	Penicillin	1302 ± 4.93 (148)	7.5 ± 0.15
8	EtBr-2	30 min	Kanamicin	995 ± 8.42 (113)	7.7 ± 0.19
9	EtBr-7	30 min	Penicillin	1042 ± 4.47 (118)	7.9 ± 0.25
10	EtBr-11	1 h	Kanamicin	1198 ± 3.58 (136)	8.1 ± 0.23
11	EtBr-14	30 min	Penicillin	1187 ± 5.61 (135)	7.7 ± 0.18
12	EtBr-16	1 h	Penicillin	1282 ± 6.52 (145)	6.9 ± 0.20
13	EtBr-19	1 h	Kanamicin	984 ± 4.56 (112)	7.8 ± 0.16
14	EtBr-22	30 min	Penicillin	1354 ± 4.54 (154)	7.6 ± 0.18
15	EtBr-24	1 h	Kanamicin	1068 ± 6.24 (121)	7.9 ± 0.21
16	EtBr-28	1 h	Penicillin	1294 ± 5.14 (147)	8.0 ± 0.17
17	MNNG-2	1 h	Penicillin	1045 ± 4.27 (118)	7.5 ± 0.18
18	MNNG-6	30 min	Penicillin	1283 ± 5.45 (145)	7.8 ± 0.24
19	MNNG-7	1 h	Kanamicin	1083 ± 3.24 (123)	8.3 ± 0.18
20	MNNG-10	30 min	Penicillin	997 ± 6.25 (113)	7.5 ± 0.20
21	MNNG-12	1 h	Kanamicin	1138 ± 8.54 (130)	8.1 ± 0.15
22	MNNG-14	1 h	Penicillin	1173 ± 7.42 (132)	7.6 ± 0.14
23	MNNG-19	1 h	Penicillin	1298 ± 5.32 (147)	6.9 ± 0.17

The relative gentamicin production (%) compared with the production of the parental strain (taken as 100%) is given in parentheses.
[a] Mean values ± S.D. ($n = 6$).