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Effect of Ethidium Bromide (EB) and N-methyl-N'-Nitro-N-Nitrosoguanidine (NTG) on Metabolic Activities of *Streptomyces* Strain N-404

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A B S T R A C T

In this report, an attempt to study the effect of production of inhibitory bioactive compounds and fiber hydrolytic enzymes was demonstrated by mutagenic agent treatment analysis. The effects of two chemical mutagenic agents [N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (65-500 µg/ml) and Ethidium Bromide (EB) (5-25 µM)] on the loss of enzymes and antibiotic activities and generation of bald mutants (no aerial mycelia) in *Streptomyces* strain N-404, were observed. Generation of bald mutants was observed with treatment with 65 µg/ml of NTG and 10 µM EB after 5 and 14 days, respectively. Production of antibiotics and fiber hydrolytic enzymes (amylase, cellulase, and xylanase) was not eliminated, but noticeable reduction of these activities in the mutant strain was detected.

Introduction

Members of the genus *Streptomyces* produce two–thirds of the naturally occurring antibiotics worldwide (Berdy, 1995), with streptomycetes being the best known enzyme producers (Vinogradova and Kushnir, 2003). These Gram-positive soil bacteria undergo a complex cycle of morphological differentiation. They produce extensive branching of vegetative hyphae and aerial mycelia on solid media which give the colony its leathery or powdery appearance. Upon maturity, aerial mycelia form chains of spores (Anderson and Wellington, 2001). Several studies have been conducted to investigate the effect of physical and chemical mutagenic agents on the alteration in the regular colony morphology of *Streptomyces* spp. and how this change in morphology is linked with the production of secondary metabolites. These agents are also responsible for the production of bald mutants (loss of formation of aerial mycelia). For example, treatment of *Streptomyces* species with ethidium bromide (EB), acriflavine (AF), or acridine orange (AO) can generate "bald" mutants which have lost the ability to sporulate and may also lack the ability to synthesize off-flavor compounds (Redshaw *et al.*, 1976; Redshaw *et al.*, 1979). Saadoun *et al.*, (1998) showed the formation of bald mutants and loss of aerial mycelia

(amy$^-$) after treatment of *Streptomyces halstedii* and *Streptomyces violaceusniger* with EB and NTG.

In this communication, the effects of two curing agents [Ethidium Bromide (EB) and N-methyl-N'-nitro-N-nitrosoguanidine (NTG)] on growth response, production of enzymes and antibiotics as well as loss of its morphology and aerial mycelium by *Streptomyces* strain N-404 is reported. Therefore, the purpose of this work is to ascertain whether enzymes/antibiotics production is affected after mutation treatment.

Materials and Methods

Streptomyces spp. (N-404 strain) an antibiotic active-producing isolate was used in this study. The isolate was previously recovered from soil in the Northern Jordan and shown to be active against multi-drug resistant pathogens.

Preparation of spore suspension

Spore suspension of *Streptomyces* strain N-404 was freshly prepared or frozen in 20% glycerol according to Hopwood (1985). Culture of N-404 strain was grown on Hickey-Tresner (HT) (Hickey and Tresner 1952) agar (pH 7.3) for 10 days at 28°C. The spores were harvested by scraping, suspended in 0.1% Tween 80, then vortexed for 9 min at room temperature. The suspension was filtered through glass wool and Whatman #1 filter paper, and the spore pellet was suspended in 20% glycerol.

Protoplast preparation

Briefly, 0.1 ml of fresh or frozen spore suspension of *Streptomyces* N-404 strain was inoculated into 25 ml yeast extract malt extract (YEME) broth, incubated at (30°C/200rpm) for 36-40 hr, then centrifuged at (30°C/200rpm) for 10 min. Pellet was suspended in 15 ml of 10.3% sucrose, and then centrifuged at (30°C/200rpm). After that, the pellet was re-suspended in 4 ml of lysozyme solution (2 mg/ml) in protoplast buffer, followed by incubation at 30°C/20 min, and then by addition of 5 ml protoplast buffer. Finally, the protoplast buffer was filtered through cotton wool and centrifuged at (3000rpm/7min), and then the pellet was suspended in protoplast buffer and kept in eppendorf tubes -70°C (Blatz and Matsushima, 1983).

Treatment with chemical mutagens

Stocks for the chemical mutagenic agents [N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and Ethidium Bromide (EB) were prepared in 0.05M Tris Malieic (TM) buffer, and the pH was adjusted to 8. Crystals of both mutagenic agents were vigorously vortexed until dissolved, filtered through Millipore size filter (0.45 µm), then incubated at 30°C for 1-2 hours (Delic *et al.*, 1970).

Disrupting enzymes/antibiotic activities and alteration in morphology was attempted by inoculating the spore suspension into 100 ml flasks containing 25 ml HT broth. The mutagenic agents were added to *Streptomyces* N-404 cultures to achieve the required concentration (65–500 µg/ml) and 5-25 µM for [N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and Ethidium Bromide (EB), respectively. Control flasks receive no mutagenic agents. Broth cultures were incubated at 28 °C/200 rpm for 14 days. After observing growth at 1, 2, 3, 5, 10, and 14 days intervals, serial dilutions were plated onto HT agar medium, and plates were incubated for 48 hr at 28°C. Presumptive aerial mycelium-negative (amy$^-$) colonies were picked aseptically and re-plated onto HT agar medium under the same conditions.

Testing for enzymatic activities by agar diffusion method

The wild type and mutated culture of *Streptomyces* N-404 strain was tested for amylase, cellulase, and xylanase enzymes production. The isolate was cultured on agar media having 1% of each carbon sources (Nanmori *et al.,* 1990).

Testing for amylase- production

Pure isolate of *Streptomyces* N-404 is cultured on starch casein nitrate agar (SCNA) and incubated at 28° C for 3-4 days. The plates were then flooded with Gram's iodine solution and left for 5 minutes, then washed with distilled water. Bacterial colonies producing amylase show clear zone against black color of stained starch (Santos and Martins, 2003).

Testing for cellulase- production

Pure isolate of *Streptomyces* N-404 is cultured on cellulose agar of the following composition (g/L) yeast extract: 1 g; CMC (carboxy methyl cellulose): 10 g; KH_2PO_4: 4 g; NaCl: 2 g; $MgSO_4.7H_2O$: 1 g; $MnSO_4$: 0.05 g; $FeSO4.7H_2O$: 0.05 g; $CaCl_2.2H_2O$: 2 g; NH_4Cl: 2 g and agar: 20 g; pH 7-7.2. Plates were incubated at 28°C for 4 days and then flooded with 0.1% Congo red and left for 15 – 20 min, washed with 1ml NaCl (1M) and left for 15 min. Bacterial colonies producing cellulase show clear zones against red color of non–hydrolyzed media (Sharma *et al.,* 1990). Positive isolates were tested again to confirm cellulase production.

Testing for xylanase-production

Pure isolate of *Streptomyces* N-404 is cultured on xylan agar of the following composition (g/L) yeast extract: 1 g; xylan: 10 g; KH_2PO_4: 4 g; NaCl: 2 g; $MgSO_4.7H_2O$: 1 g; $MnSO_4$: 0.05 g; $FeSO4.7H_2O$: 0.05 g; $CaCl_2.2H_2O$: 2 g; NH_4Cl: 2 g and agar: 15 g; pH 7-7.2. Plates were incubated at 28°C for 4 days. The plates were then flooded with absolute ethanol (99%) and left for 1 hour at room temperature. Colonies producing xylanase enzyme show clear zones against an opaque color of non-hydrolyzed media (Priest, 1985). Positive activity is tested again for confirmation.

Antimicrobial activity by agar disc diffusion method

This was tested by the Bauer–Kirby method (Bauer *et al.,* 1966) against *Staphylococcus aureus* and *Escherichia coli*. Each tested microbe was grown in 250 ml Erlenmeyer flask containing 50 ml of nutrient broth (Himedia/India) (pH 7.2) with overnight shaking at 100 rpm and 37 °C. Turbidity of organisms in the broth was adjusted to be equal to or greater than 0.5 McFarland turbidity standards (1.5×10^8 cfu/ml). The test organisms were homogeneously inoculated by a sterile cotton swab on the surface of two freshly prepared Mueller-Hinton agar (Himedia, India) then 3 agar discs (5 mm in diameter) were cut out from each wild type and mutagenic agent treated *Streptomyces* N-404 culture that has been grown on HT agar medium at 28 °C± for 10 days (Saadoun *et al.,* 1998); and then transferred by a flame-sterilized needle to be placed onto the surface of Mueller-Hinton agar plates. Plates that were seeded only by the tested pathogens were considered as negative controls. Plates were then incubated at 28 °C± 1. Inhibition zones were visually detected after 24 h.

Results and Discussion

Attempts were made to generate bald mutants by treatment of *Streptomyces* N-404 protoplasts with dyes. When the mutated *Streptomyces* N-404 protoplasts were

compared with the untreated ones, data indicated a weak growth response in HT broth even at the lowest applied concentration of each of EB (5 µM) and NTG (65 µg/ml) (Table 1). Growth response of the mutated protoplasts was followed in HT broth and our observations indicated the formation of long threads with no spherical shape beads (Fig. 1A). These mutants generated in the submerged cultures after treatment with mutagenic agents were called in this study as "Submerged mutants" (SMs) and characterized by irregular growth as has often been reported in *Streptomyces* species (Yamazaki *et. al.*, 2003a, 2003b; Kato *et. al.*, 2005). On HT agar surface, the parental *Streptomyces* N-404 wild-type colonies were characterized by long chains of spores and white powdery appearance (Fig. 2A). However, colonies derived by treatment of *Streptomyces* N-404 protoplasts with 5 µM (EB) for 14 days appeared soft and the formation of aerial mycelium is critically affected that resulted in generation of bald mutants and loss of aerial mycelia (amy^{-}) (Fig. 2B). This loss of aerial mycelia after dye treatment has often been reported in *Streptomyces* species (Ikeda *et. al* 1981; Hopwood *et al.*, 1973; Redshaw *et al.*, 1976; Sermonti *et al.*, 1980; Saadoun *et al.*, 1998). Redshaw *et al.*, (1979) suggested that treatment of *Streptomyces* species with mutagenic agents such as ethidium bromide (EB), acriflavine (AF), and acridine orange (AO) may responsible for the production of bald mutants (loss of formation of aerial mycelia).

Streptomyces N404 strain showed the potential to degraded starch, cellulose, and xylan (Table 2) as indicated by the clear zones around the colonies. Data indicated the wild type and mutated colonies produce the three tested enzymes; amylase, cellulase, and xylanase (Fig. 3). The three tested enzymes were maintained by both bald and SMs after treatment with the dyes.

Streptomyces N-404 exhibited an antibiotic activity against *Staphylococcus aureus* and *Escherichia coli* with 9 mm inhibition zone diameter (Table 2) and (Fig. 4). Production of inhibitory metabolites by the bald mutants generated in this study was not eliminated, but was reduced (Fig. 4C), and this may explain the linkage gene of these two processes and the involvement of linked genes on the chromosome (Saadoun *et al.*, 1998).

Table.1 Generation of bald mutants and growth response of *Streptomyces* N-404 strain in HT broth after treatment with EB and NTG chemical mutagenic agents

Time (Days)	Mutagenic Agent								
	EB (µM)				NTG (µg/ml)				
	0	5	10	25	0	65	130	250	500
1	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND
2	+[b]	ND	ND	ND	+	ND	ND	ND	ND
3	++	ND	ND	ND	++	ND	ND	ND	ND
5	+++	ND	ND	ND	+++	+	ND	ND	ND
10	+++	ND	ND	ND	+++	ND	ND	ND	ND
14	+++	+	+	ND	+++	ND	ND	ND	ND

[a] ND: Not detected.

[b] Growth response: +: Weak; ++: Moderate; +++: Strong

Table.2 Inhibitory metabolites and degradation activities of wild type and mutant *Streptomyces* N-404

Culture	Enzyme			Antibiosis (mm Inhibition Zone Diameter)	
	Amylase	Cellulase	Xylanase	*S. aureus*	*E. coli*
Wild type	+	+	+	9	9
Bald mutant	+	+	+	9 (No Clear Zone)	9 (No Clear Zone)
SMs	+	+	+	9	9

Fig.1 *Streptomyces* N-404 strain grown in HT broth and treated with mutagenic agents. A: Submerged mutants (SMs) after treatment with 65 µg/l (NTG); B: Wild type

Fig.2 Treatment *Streptomyces* N-404 colonies on HT agar surface with ethidium bromide (EB). (A) Wild type; (B) Bald mutant after treatment with 5 µM EB for 14 days

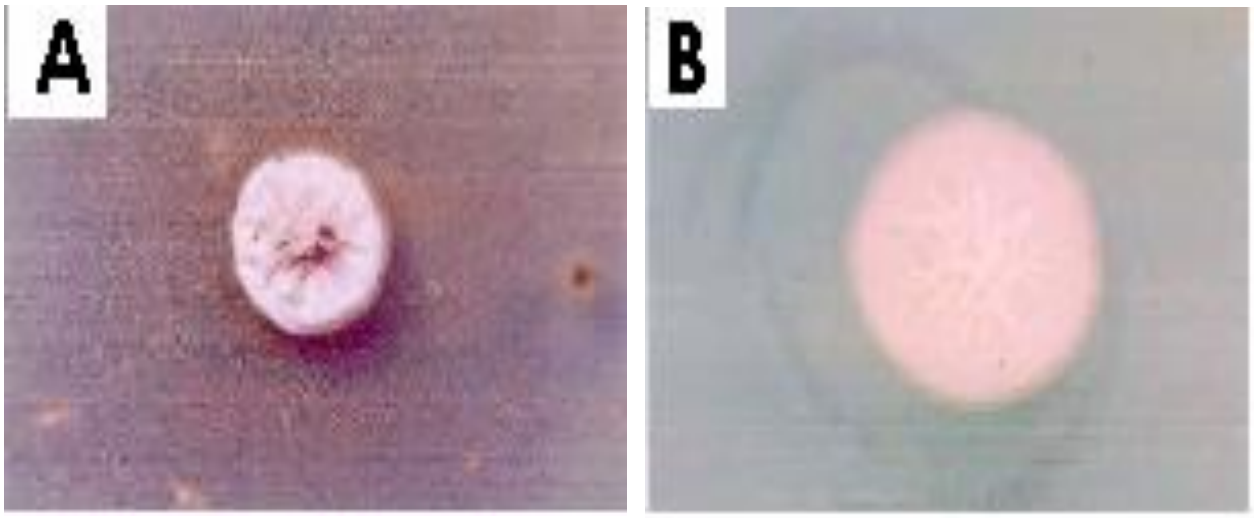


Fig.3 Testing for (1) cellulase; (2) xylanase; and (3) amylase production by *Streptomyces* N-404 cultures treated with 5 µM EB. A: Wild type; B: Bald mutants, C: Submerged mutants (SMs)

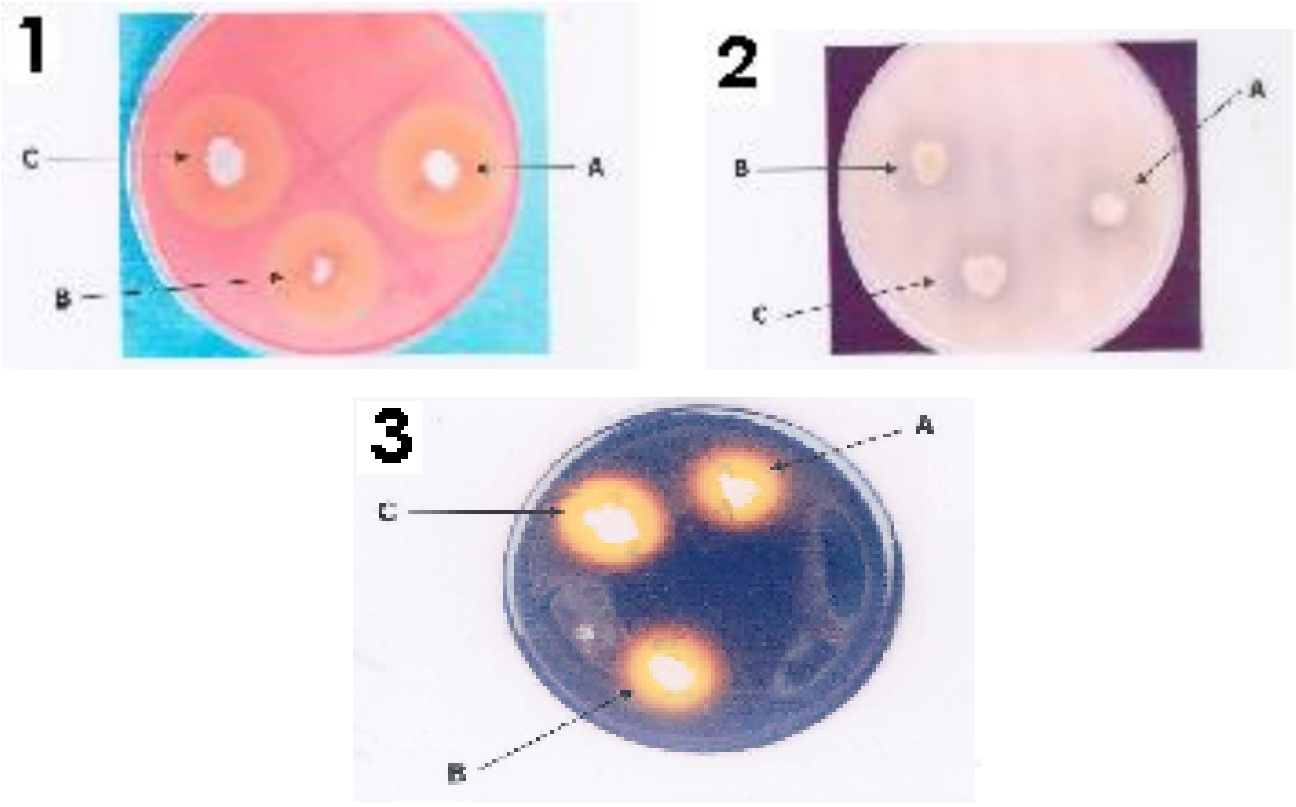
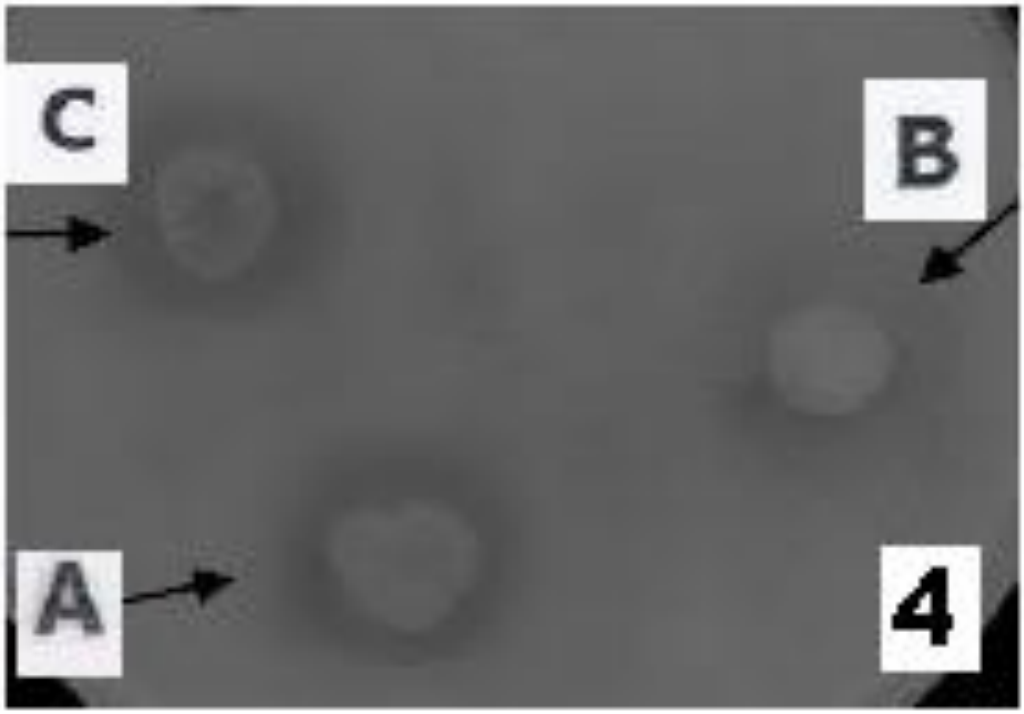


Fig.4 Testing for antibiosis by *Streptomyces* N-404 cultures against *Staphylococcus aureus*. A: Wild type; B: Bald mutant; C: Submerged mutants (SMs)



The processes of secondary metabolism (antibiotic production) and morphological differentiation (aerial mycelia formation and subsequent spore formation) in streptomycetes have been "linked" because both phenomena apparently occur simultaneously during the life cycle of these organisms. The coincidence of sporulation with the one onset of secondary metabolite production has been described by some as correlative relationship but not an interdependent one (Bu'lock, 1961; Schaeffer, 1969). Our results indicated that inhibitory metabolites production was not completely lost in the bald and SMs mutants generated by treatment with EB and NTG.

This would suggest that antibiotic production is likely chromosomally-encoded in this *Streptomyces* isolate used in this study. However, mutagenesis of a chromosomal gene could be responsible for this loss, since the dyes used could cause chromosomal aberrations.

This study showed the formation of bald mutants and loss of aerial mycelia (amy^-) after treatment of *Streptomyces* species with EB and NTG with maintaining the enzyme and antibiotic production activity.

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