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Screening of Mutant Strain *Streptomyces mediolani* sp. AC37 for (-)-8-O-Methyltetrangomycin Production Enhancement

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Streptomyces mediolani **sp. AC37 was isolated from the root system of higher plant *Taxus baccata* and produced metabolite identified as (-)-8-O-methyltetrangomycin according to LC/MS/MS analysis. In our screening program for improvements of bioactive secondary metabolites from plant associate streptomycetes, mutation was used as a tool for the induction of genetic variations for selection of higher (-)-8-O-methyltetrangomycin producers of isolates. *S. mediolani* sp. AC37 was treated with UV irradiation and chemical mutagenic treatment (N-nitroso-N-methyl-urea). The radical scavenging and antioxidant capacity of (-)-8-O-methyltetrangomycin and extracts isolated from mutants were tested using EPR spin trapping technique and ABTS$^{\bullet +}$ assay. Comparison of electron microscopic images of *Streptomyces* sp. AC37 and mutant strains of *Streptomyces* sp. AC37 revealed substantial differences in morphology and ultrastructure.**

Keywords: *Streptomyces mediolani*, (-)-8-O-methyltetrangomycin, mutation, N-nitroso-N-methyl-urea, EPR spectroscopy, sulphate radical anion, antioxidant

Introduction

Achieving higher production of bioactive metabolites is an important factor in the pharmaceutical industry. High-production strains prepared using mutagen effects show several-times higher production of desirable substances as original parental strains. Thus, in many cases, this procedure is appropriate for the production improvement of low-production microorganism strains.

Improvement of productivity of commercially viable microorganisms is an important field in microbiology, particularly since wild-type strains isolated from nature usually produce only low levels (1–100 μg/ml) of antibiotics. A great deal of effort and resources must therefore committed to improve antibiotic-producing strains with an objective of meeting commercial requirements (Tamehiro *et al.*, 2003). Current methods of improving the productivity of industrial microorganisms range from the classical random approach to using highly rational methods, such as metabolic engineering. Occasionally, product regulatory mutants obtained in basic genetic studies are found to be altered in colonial morphology. These morphological mutants are very important in the strain improvement activities (Adrio and Demain, 2006). Although classical methods are still effective in obtaining highly productive strains, even without using genomic information or genetic tools, these methods are always time and resource consuming (Zhang *et al.*, 2002; Khaliq *et al.*, 2009).

(-)-8-O-Methyltetrangomycin (8-OMTGM) is an angucycline antibiotic produced by *Streptomyces* sp. AC113 (Maruna *et al.*, 2010), which displays antibacterial activity (MIC against *Listeria monocytogenes* of 14.5 μg/ml) and cytotoxicity (IC_{50} 7.13 μg/ml for B16 cell line and IC_{50} 66.9 μg/ml for HT-29 cell line). This compound has also been previously isolated by Maehr *et al.* (1982), Gilpin *et al.* (1989) from *Streptomyces* sp. MM47755 and Abdelfattah *et al.* (2003) from *Streptomyces* sp. GW19/1251. More recently, the angucycline antibiotics have been attracting considerable attention because of their diverse biological activities as well as unique structural features; that is, the chromophore is a benz[α]anthracene, which varies in the oxidation state and/or the location of the oxygen functionalities and is often armed with the C-glycoside (Takashi, 2000). Therefore, this group of antibiotics is, after tetracyclines and anthracyclines, the third class of natural antibiotics featuring a carbotetracyclic skeleton and displays a broad spectrum of biological activities (Rohr and Thiericke, 1992; Krohn and Rohr, 1997) including antitumor (Antal *et al.*, 2005; Korynevska *et al.*, 2007) and antibacterial activities (Abdelfattah *et al.*, 2003; Antal *et al.*, 2005), as well as platelet aggregation inhibition (Kawashima *et al.*, 1989; Omura *et al.*, 2001).

Mutations of the genes for methylation, regulation and glycosylation can provide molecules with advantageous characteristics for industrial production, often increasing the yield of the product. Due to the high frequency of *Streptomyces* phenotypes with low productivity and viability (Volff and Altenbuchner, 1998), a constant selection of stable populations is necessary.

The importance of compounds bearing antioxidant activity lies in the fact that they are highly effective against damage caused by reactive oxygen species (ROSs) and oxygen-derived free radicals, which contribute to a variety of patho-

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logical effects, for instance, DNA damages, carcinogenesis, and cellular degeneration (Huang *et al*., 2007; Seifried *et al*., 2007). Natural antioxidants are commonly found in medicinal plants, vegetables, and fruits. However, it has been reported that metabolites from endophytes can be a potential source of novel natural antioxidants. Liu and co-workers evaluated the antioxidant activity of an endophytic *Xylaria* sp. isolated from the medicinal plant *Ginkgo biloba* (Liu *et al*., 2007). The compounds cladoniamides (A–G) are the potent free radical scavengers isolated from the actinobacteria *Streptomyces uncialis* (Williams *et al*., 2008).

The aim of this work was to apply an efficient screening method to obtain better (-)-8-O-methyltetrangomycin producers through induced mutants of *Streptomyces* sp. AC37. Moreover, we proposed to compare the antioxidant capacity on the grounds of (-)-8-O-methyltetrangomycin production between the original strain *Streptomyces* sp. AC37 and the extract of the best mutants producing this substance.

Materials and Methods

Microorganisms

Gram-positive filamentous bacteria were isolated from the root system of higher plant *Taxus baccata,* from the Bratislava region, Slovakia. The culture was identified as *S.* sp. AC37, which is closely related (99%) to *S. mediolani* by 16S rRNA analysis. The gene sequence was submitted to NCBI GenBank with accession code FJ001756. *S. griseus* is considered to be later heterotypic synonymus of *S. mediolani* (Rong and Huang, 2010). The strain was cultivated and maintained on Actinomycete Isolation Agar (AIA) (Biomark, India) at 28°C for 11 days.

Chemicals

The spin trapping agent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; Aldrich) was distilled before application and saved under argon at -18°C. Radical cation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS; Sigma, USA) was prepared by dissolving 17.2 mg of ABTS and 3.3 mg of $K_2S_2O_8$ (Aldrich) in 5 ml of distilled water as described in Re *et al.* (1999). Prepared solutions of $ABTS^{•+}$ were diluted immediately before measurements in order to obtain the optimal concentration for UV/V is experiments (~80 μM). Dimethyl sulfoxide (DMSO, SeccoSolv® max. 0.025% H_2O) was obtained from Merck (Germany).

The samples were then fixed with 2% glutaraldehyde (Sigma) in cacodylate buffer (Merck) (in mM: 150 Na-cacodylate, 2.0 $CaCl_2$, pH 7.3). Mutagenesis was carried out by N-nitroso-N-methyl-urea (NNMU) (Sigma). Extraction and elucidation was prepared by chloroform, methanol, ethyl acetate, n-hexan (Merck). (-)-8-O-Methyltetrangomycin isolated from *Streptomyces* sp. AC113 by Maruna *et al.* (2010).

Electron microscopy

Bacterial suspension of *Streptomyces* sp. AC37 and the mutant AC37-9M-16 were centrifugated at 600 rev/min for 5 min in the centrifuge K 26 (Janetzki, Poland) in Ringers solution (Merck) with 2% glutaraldehyde. The samples were then fixed with 2% glutaraldehyde in cacodylate buffer (in mM: 150 Na-cacodylate, 2.0 $CaCl_2$, pH 7.3) for 1 h. After the postfixation with 1% osmium tetroxide in cacodylate buffer for 45 min at room temperature, the samples were stained with 1% aqueous solution of uranyl acetate. Subsequent to the dehydration in graded ethanol series and acetone, the samples were embedded into Durcupan (Fluka AG, Switzerland). Ultrathin (58–60 nm) sections were cut by Power-Tome MT-XL ultramicrotome (RMC/Sorvall, Tucson, USA), placed on copper grids covered with formwar and contrasted by lead citrate. The sections were examined with JEM 1200 electron microscope (Jeol, Japan) at 75000–200000x magnification. Images were recorded using a Gatan Dual Vision 300W CCD camera (Gatan Inc., USA).

Mutagenic treatments and monitoring

Mutagenesis by an ultraviolet radiation: The spore suspension of *Streptomyces* sp. AC37 was diluted to suitable concentration (10^7 spore/ml) and 0.1 ml of the suspension was inoculated onto the Petri dishes with AIA medium. Dishes were placed under the ultraviolet light (Sterillampe VS-310, Poland) at a distance of 22 cm from the UV lamp with power of 30 W. The lid of the inoculated plates was removed and spore suspension was exposed to UV radiation for 5, 10, 15, and 20 sec, respectively. The plates were incubated at 28°C in dark for 11 days.

Mutagenesis by N-nitroso-N-methyl-urea (NNMU): *N*-nitroso-*N*-methyl-urea (Sigma) (the final concentration 8 mg/ml) was added to the diluted spore suspension (10^7 spores/ml). The spore suspension was carefully stirred for 30 min. The spores were then collected by centrifugation, washed with sterile water and resuspended in 5 ml of sterile distilled water. The Petri dishes were inoculated with 0.1 ml of this suspension and incubated at 28°C in dark for 11 days. Each experiment was performed on two parallel Petri dishes with the derivation of measurement not higher than 10%.

Monitoring of mutagenesis was accomplished by changes in three phenotypic features: (1) morphological alterations: absence of aerial mycelia, and absence of spores; (2) absence of pigment production; (3) blocked production of 8-OMTGM.

Measurement of cell growth

Determination of dry cell mass (DCM) was carried out. Cultivation medium (10 ml) was removed and centrifugated during 10 min at 2800 rev/min. Following the centrifugation, the supernatant was separated; the biomass was washed with distilled water twice and was recentrifugated. The aqueous layer was divided and the biomass was quantitatively transferred into a previously weighed plotter and dried at 45°C during 24 h. Dry mass was determined after cooling and desiccation, and the determination continued until a constant mass was obtained.

Glucose determination

The supernatant obtained by having centrifugated 10 ml of culture medium was adequately diluted. For the analysis, we used 40 μl of the diluted sample and added to 4 ml of glucose-oxidase solution (BIO-LA-TEST® Glucose God 1500,

Lachema, Czech Republic); it was stirred and incubated at 25°C, in dark during 30 min. The absorbance was measured at 498 nm (UV/vis spectrometer Spectronic 20D Milton Roy, USA) against the distilled water solution. The glucose concentration in the medium was calculated by calibration curve method.

Fermentation conditions and preparation of extracts

Mutated spores and ground control spores were inoculated on a solid medium. Each obtained monocolony was transferred to a tube slant. The spores that arose after 11 days of cultivation at 28°C were inoculated into a 500 ml flask with 100 ml of Actinomyces Broth (AIB) medium. The vegetative inoculum was grown on an AIB (Biomark, India) in 500 ml Erlenmeyer flasks containing 100 ml of medium at 28°C in rotary shaker 180 rev/min, in the dark for 48 h. Seed culture at the rate of 10% was transferred into fermentation broth containing (g/L): glucose 20; arginine 1; glycerol 12.5; $K_2HPO_4{\cdot}3H_2O$ 1.3; NaCl 1; $MgSO_4{\cdot}7H_2O$ 0.5; acid benzoate 0.05; phenylalanine 0.03; all dissolved in distilled water and pH adjusted to 7.5 and incubated on a rotary shaker (180 rev/ min) at 28°C, in the dark for 120 h. The supernatant culture (10 ml) was obtained after centrifugation (2800 rev/min) at 10 min. The supernatant was extracted first with *n*-hexane (5 ml) and subsequently with ethyl acetate (5 ml) and concentrated under reduced pressure to yield the crude extract.

Analytical measurement and determination of 8-OMTGM

Isolated crude extracts were dissolved in chloroform (Merck) and the volume of 40 µl was applied into silica gel 60 F_{254} thin layer chromatography plates (Merck). TLC analyses were carried out in the solvents as follows: Solvent A, dichloromethane: methanol (10:0.6, v/v); solvent B, chloroform: acetone, (65:35, v/v). After TLC running and development, the TLC plate was evaluated in UV light λ=254 nm and λ=366 nm. TLC analysis of the ethyl acetate extract of *Streptomyces* sp. AC37 as well as the methanolic solution of 8-OMTGM standard was carried out using solvent A by spotting on the start line of a 0.25 mm (10×20 cm) silica gel plate. 8-OMTGM was detected using a spray reagent consisting of 1% vanillin (w/v) in sulphuric acid after gentle heating (Cardellina, 1991). It appeared as a bluish spot which turned dark yellow after 24 h. The (-)-8-*O*-methyltetrangomycin spot was identified by comigration with an authentic sample of (-)-8-*O*-methyltetrangomycin as a standard (Maruna *et al.*, 2010).

The cultivation medium extracts were dissolved in methanol (Merck) and applied in HPLC. For the analysis, we used the mobile phase of methanol : acetonitrile : water (25 mM KH_2PO_4). The proportion of these two phases changed during the analysis from the initial value of 1:10:89 to 1:89:10 in the 20th min at the end of the analysis. Used column: LiChroCART® 250-4 (length 25 cm), mobile phase flow: 1 ml/min, UV detector wave length: 228 nm.

LC-MS/MS analysis

Analyses were performed on an Agilent (Germany) 1100 series HPLC equipped with Supelco (USA) column ABZ+Plus 150 mm × 4.6 mm, 3 µm particle size. The injection volume was 10 µl and the elution flow rate 1 ml/min. Samples were separated using gradient from acetonitrile/water (10/90, v/v) with 0.2% of formic acid to acetonitril with 0.2% of formic acid in 36 min. Detection was performed using a Bruker (Germany) LC/MSD Trap VL. The instrument was set for ESI in positive and negative mode as follows: Nebulizing gas pressure 40 psi, flow rate 10 L/min, drying temperature 350°C. The capillary voltage was set at 4 kV. The instrument was set for APCI in positive and negative mode as follows: Nebulizing gas pressure 40 psi, flow rate 5 L/min, drying temperature 325°C, vaporizer temperature 400°C. The corona needle was set at 10000 nA. The system was optimized for target mass 300 daltons and scan range from 50 to 1000 daltons. The system was set in both types of measurements to make fragmentation from two most intense ions at fragmentation energy 1 V.

Radical scavenging capacity determined by EPR spectroscopy

The ability of extract isolated from mutant AC37-9M-16 (E2), extract isolated from *Streptomyces* sp. AC37 (E1), and 8-OMTGM dissolved in DMSO solvent to terminate reactive sulfate radical anions was investigated by EPR spin trapping technique using DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) (Zalibera *et al.*, 2009). The solutions for EPR measurements (200 µl of sample dissolved in DMSO; 25 µl of DMPO in DMSO, $c_{0,\mathrm{DMPO}}$=0.2 M; 25 µl of $K_2S_2O_8$ in water, $c_{0,\mathrm{K2S2O8}}$=0.01 M) prepared immediately before measurements were carefully mixed by a slight air stream and immediately transferred to a small quartz flat cell (WG 808-Q, Wilmad-LabGlass, USA) optimized for the TE_{102} cavity of an EMX X-band EPR spectrometer (Bruker, Germany). The ER 4111 VT temperature unit (Bruker) was used for temperature regulation. Special attention was focused on the identical positioning of the cell in the EPR cavity and the proper spectrometer tuning in all experiments. The sets of 20 individual EPR spectra (EPR spectrum represents three 22-sec scans) were monitored *in situ* at temperature of 60°C (Fig. 5), and the measurements were performed in duplicate for each sample. In reference experiments, the samples were replaced by DMSO solvent. Following the current trends in the antioxidant activity research, the analogous EPR spin trapping experiments were carried out in the presence of various concentration of trolox (water soluble α-tocopherol analogue) in DMSO, and the data obtained were used to evaluate the radical scavenging capacity of sample (RSC) representing mmol of trolox per 1 g of dry sample (Zalibera *et al.*, 2009).

The EPR spectrometer settings were: microwave frequency, 9.448 GHz; microwave power, 10.03 mW; center field, 335.25 mT; sweep width, 8 mT; gain, $5.02{\times}10^4$; modulation amplitude, 0.1 mT; scan, 20.97 s; time delay, 1.03 s; time constant, 40.96 ms; number of scans, 3. Detected EPR spectra were analyzed and simulated by the Bruker software WinEPR and SimFonia and the Winsim2002 software free available from the website of National Institute of Environmental Health Sciences (NIEHS) (http://epr.niehs.nih.gov/) (WinSIM, NIEHS, Research Triangle Park, NC USA 27709, 2002).

Antioxidant capacity determined by TEAC approach

The TEAC method was used to determine antioxidant capacity of isolated extracts E1–E2 and 8-OMTGM dissolved in DMSO. This approach is based on termination of radical cation $ABTS^{\bullet+}$ possessing characteristic absorption band in electronic spectrum at 730 nm (ε_{730} =14.7 mM^{-1} cm^{-1}, Arts *et al*., 2004). In our UV/vis investigations, 200 µl of sample in DMSO was placed in a quartz cell (optical path length 1 cm) and 2.8 ml of aqueous solutions of $ABTS^{\bullet+}$ was added rapidly. Ten seconds after mixing both solutions, UV/vis spectrometer (UV-3600 Shimadzu, Japan) starts to monitor the absorbance change at 730 nm during 600 sec. In the reference experiments, the sample solutions were replaced by DMSO solvent. Analogous spectrophotometric experiments were performed with trolox in DMSO, and the decrease in molar amount of $ABTS^{\bullet+}$ obtained after 610 sec was used to construct calibration curve. The TEAC values of samples were evaluated in mmol of trolox per 1 g of dry sample.

Results

Mutant screening

The traditional method of secondary metabolite yields improvement is mutagenesis and fermentation analysis (Baltz, 1986). In this work, the strain *Streptomyces* sp. AC37 was mutated, induced mutants were isolated and 8-OMTGM production was analyzed by TLC and quantified by HPLC. At the same time, we carried out a comparison between the original strain and the mutant with a major capacity of 8-OMTGM production for possible morphological changes.

The original culture *Streptomyces* sp. AC37 was diluted 1:10 in a stepwise manner to give a series of solutions with predetermined dilution ratio (10^{-1}–10^{-7}), previously 0.2 ml of each solution with varying concentration was plated on a solid medium in Petri dishes. After incubation, these samples were mutated by UV radiation during 5, 10, 15, and 20 sec and incubated at 28°C for 11 days. The amount of 2,450 monocolonies were counted, from which 100 were randomly chosen. Following the mutation and submerse cultivation, we chose two different strains from the mutated cultures on the grounds of 8-OMTGM production analyses: mutant *Streptomyces* sp. AC37-9M producing brown pigments onto the medium and substrate mycelia and mutant *Streptomyces* sp. AC37-3M with pink pigments production. Both strains were exposed to another series of mutations by means of UV radiation, and afterwards 3 mutants with the best metabolites' production were selected: mutant AC37-9M-16, AC37-9M-24, and AC37-9M-22. From the mutants AC37-3M, we chose the particular mutant AC37-3M-12, which was subsequently mutated by a chemical mutagen, N-nitroso-N-methyl-urea. The original colonies of *Streptomyces* sp. AC37 (Fig. 1) (A) white and circular colonies with wrinkled surface and raised center, after the UV mutation, the morphological change in the colonies was observed, as shown in Figs. 1B–1D. The morphologies of the colonies were divided into three types: (1) rose, puffy, and circular colonies; (2) white, puffy, circular, raised center, and expelled green contents; and (3) beige, with wrinkled surface and raised center. Figs. 1E–1G shows morphologies of the three types of colonies after the NNMU mutation: (4) white, circular, and crateriform colonies; (5) white, flat, wrinkled surface and non-circular form with irregular border; (6) white, circular, crateriform colonies. *Streptomyces* sp. AC37 had a diameter of 4 mm; however, the colonies of mutants had a diameter of 6–8 mm, and the spores of all the three types of the colonies were abundant.

The screening of better 8-OMTGM producers was based upon the identification and selection of dominant morphological features (such as production of aerial mycelium, spore formation and pigment production) of the induced mutants of *Streptomyces* sp. AC37.

Ultrastructural characteristics of *Streptomyces* sp. AC37 and the mutant strains

Comparison of electron microscopic images of *Streptomyces* sp. AC37 and mutant strains of *Streptomyces* sp. AC37 revealed substantial differences in morphology and ultrastructure (Fig. 2). Wildtype bacteria had dense and rather homogenous cytoplasm with few structural features. On the other hand, the mutant bacteria that proliferated more intensely displayed more internal heterogeneities. Cytoplasm

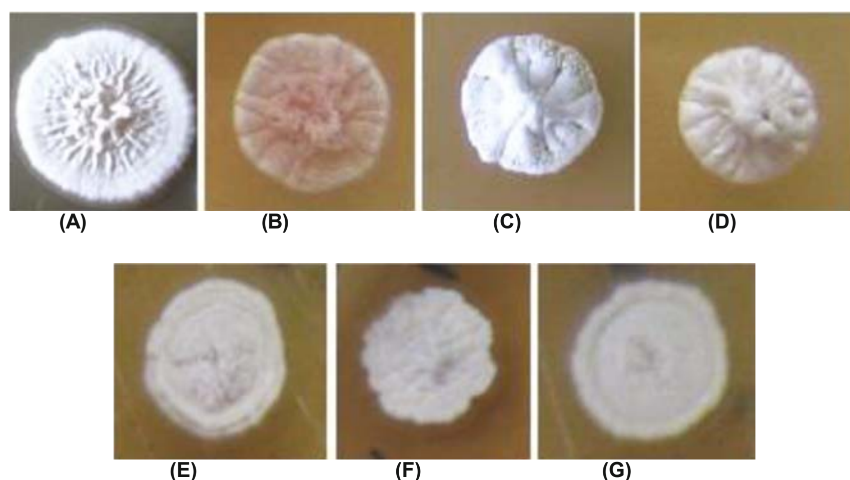

Fig. 1. Morphologies of the three types of colonies after UV mutation and NNMU mutation. (A) *Streptomyces* sp. AC37, (B) type 1 UV mutation, (C) type 2 UV mutation, (D) type 3 UV mutation, (E) type 4 NNMU mutation, (F) type 5 NNMU mutation, (G) type 6 NNMU mutation.

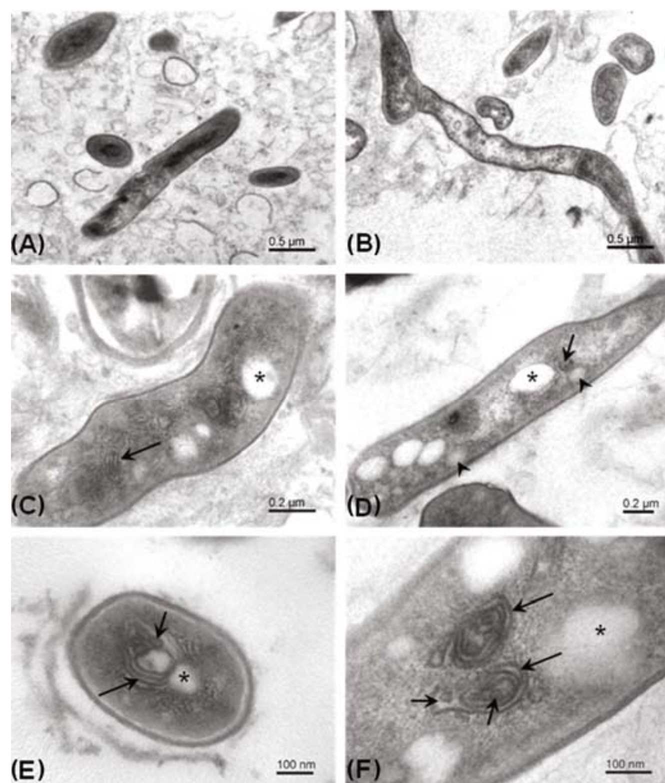

Fig. 2. Electron micrographs of *Streptomyces* sp. AC37 and the mutant strains. Typical appearance of the wild type *Streptomyces* sp. AC37 is shown in (A), (C), and (E). The mutant *Streptomyces* sp. AC37-9M-16 is shown in (B), (D), and (F). (A and B) low magnification overviews. (C and D) medium magnification; asterisks - large inclusions with light content; arrowheads - large inclusions with gray content; long arrow, nucleoid; short arrow, plasmid. [E (cross-cut) and F] high magnification with details of nucleoids (long arrows), plasmids (short arrows) and large inclusions (asterisks).

of both strains contained plasmids, ribosomes, inclusions, and nucleoids. However, they were more manifested in mutant bacteria, especially in the case of large inclusions. Large inclusions appeared either light or grey, indicating differences in their composition (Fig. 2D).

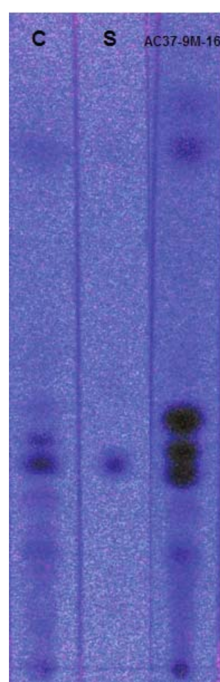

Fig. 3. TLC plate evaluated under UV light (366 nm). (dichloromethane : methanol, 1:0.6, v/v), S, standard of 8-OMTGM (0.04 ml) at a concentration of 2.0 mg/ml (Rf=0.28); C, production by control strain *Streptomyces* sp. AC37; AC37-9M-16 the best producer mutant. Volume 0.04 ml of extract dissolved in chloroform was applied into silica gel.

Identification and selection of higher 8-OMTGM producer

Upon screening of 1000 monocolonies from four mutagenic treatments, 12 mutants showed high 8-OMTGM production detected by HPLC. Mutant AC37-9M was subjected to a new UV treatment and 258 colonies were screened. More than 30 monocolonies were cultivated while submerged, resulting in the detection of a new producing mutant, called AC37-9M-16 (Fig. 3).

TLC assay showed that the mutant AC37-9M-16 improved the productions of 8-OMTGM comparing with the control. It is known that mutagenic treatments trigger genetic instability processes, increasing the frequency of mutations responsible for undesirable characteristics. Some morphological mutants were described that resulted from amplification or deletion of the genes that influence antibiotic production (Volff *et al.*, 1993).

Studies focusing on the production of antibiotics and phenomena such as strain degeneration and increase of productivity have been performed by Gravius *et al.* (1993).

Mutant AC37-9M-16 produced the highest quantity of 8-OMTGM, which was calculated using an authentic sample of (-)-8-O-methyltetrangomycin as a standard (Maruna *et al.*, 2010). Extract (E2) of this mutant strain was purified for further analysis by LC-MS/MS.

LC-MS/MS analysis was used to analyse 8-OMTGM detected in *Streptomyces* sp. AC37 and mutant AC37-9M-16. These analyses revealed an identical ion trace and fragmentation pattern for m/z 336 (M+1=337.2, M+1+22= 359.1, fragment 319.4).

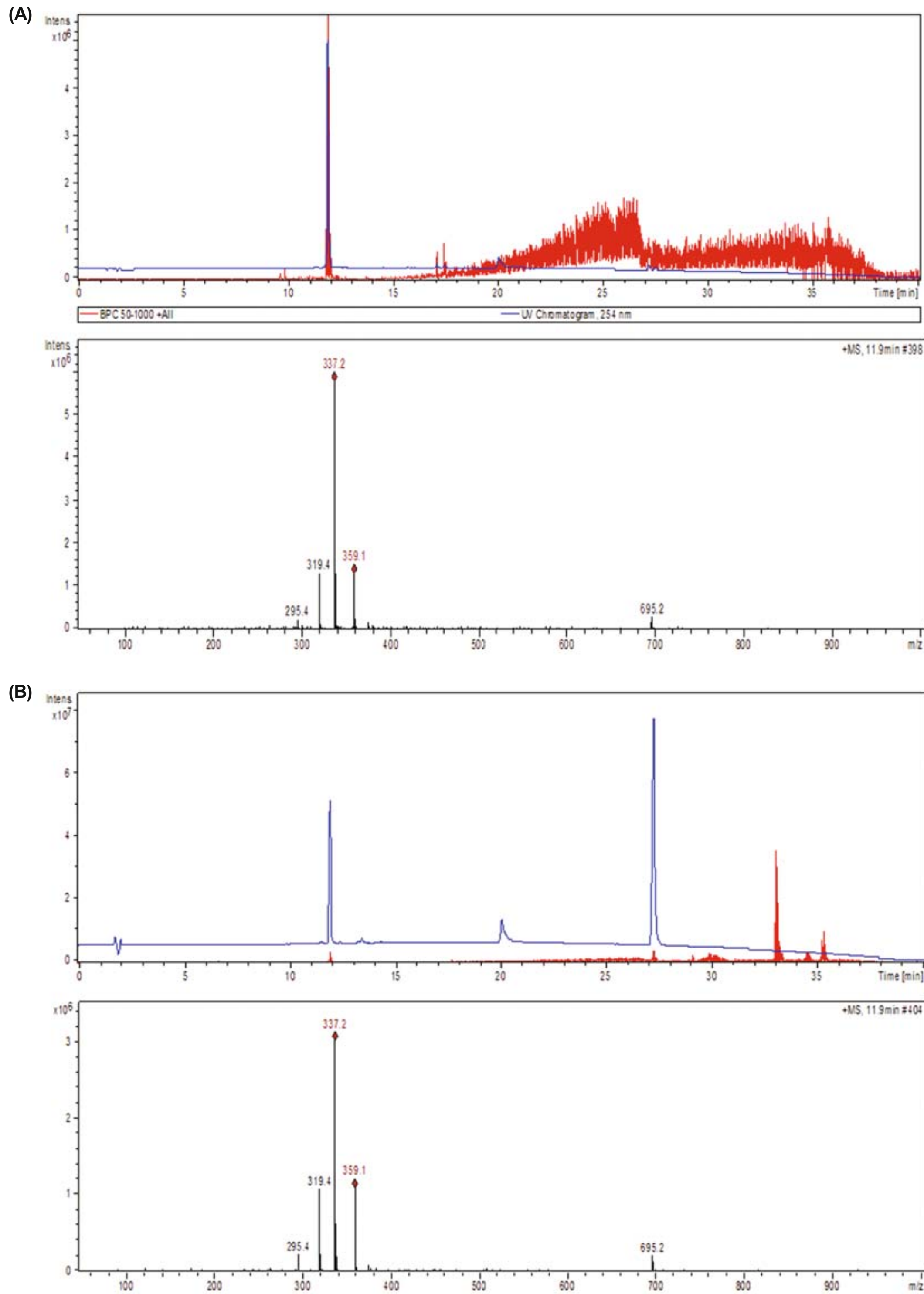

Fig. 4. (A) LC-MS/MS analysis of (-)-8-O-methyltetrangomycin. (B) MS/MS spectra sample from preparative HPLC of extract fraction from culture medium of mutant AC37-9M-16 (E2).

Cultivation of selected mutants for production of (-)-8-O-methyltetrangomycin

The productivity tests in liquid medium were carried out with the strain *Streptomyces* sp. AC37 and its mutant: AC37-9M-16. *Streptomyces* sp. AC37-9M-16 produced a larger amount of 8-OMTGM (39.85 mg/L) at 120 h, about 30-times

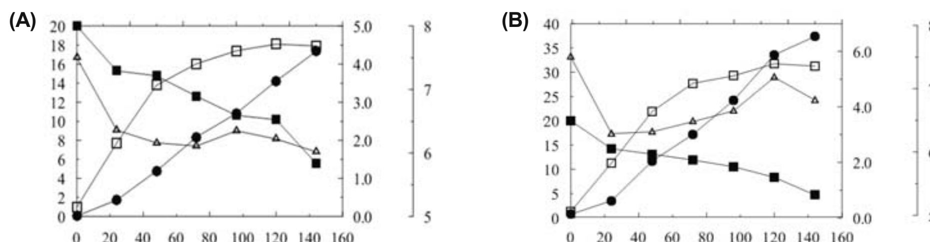

Fig. 5. **Fermentation process of *Streptomyces* sp. AC37 (A) (as a control) and mutant *Streptomyces* sp. AC37-9M-16 (B), using cultivation conditions.** 10% of vegetative inoculum (48 h), arginine-glycerol medium 100 ml in 500 ml flask on a rotary shaker (180 rev/min) at 28°C, in dark for 144 h.

more 8-OMTGM than the control strain *Streptomyces* sp. AC37 (7.66 mg/L).

From the kinetics of *Streptomyces* sp. AC37 and mutant AC37-9M-16 (Fig. 5), a very intense growth can be seen during the first 48 h of the cultivation. The biomass growth becomes slower from this phase. The stationary phase of growth was observed from the 120th h of the cultivation process. *Streptomyces* sp. AC37-9M-16 uses glucose, which is consumed intensively during the exponential phase of growth. The increase in product quantity was accompanied by faster glucose consumption. The value of cultivation medium pH decreased at first from an initial 7.5 to 6.29 in the 24th h. Between the 48th and 72nd h, it was constant and after the 96th h, it began to rise until it reached neutral value in the 120th h. At the end of the cultivation, pH value declined slightly to 6.82. The production of 8-OMTGM was noticed in the 48th h of cultivation, and its intensity increased during the 72nd and 120th h interval.

Radical scavenging capacity determined by EPR spectroscopy

One of the techniques often repeated in antioxidant activity estimation assays is based on the generation of reactive radicals in the sample with the use of an initiator, while the ability of the sample to terminate these reactive radicals is monitored by various methods. The EPR spectroscopy has one remarkable advantage among other detection methods: it provides qualitative information about the radicals present. The basic idea of an EPR spin trapping assay for RSC evaluation is the monitoring of a competitive reaction between the spin trap and antioxidants present in the sample (Zalibera *et al.*, 2009).

Sulfate radical anions generated upon thermal decomposition of $K_2S_2O_8$ represent reactive species with high reduction potential, and $SO_4^{\bullet-}$ are capable to react with a variety of organic compounds (Wardman, 1989). In DMSO solvent, these paramagnetic species are added to the double bond of DMPO spin trapping agent producing the corresponding spin adducts (Zalibera *et al.*, 2009).

The sets of 20 individual EPR spectra monitored in the presence of DMPO during heating of $K_2S_2O_8$ at 60°C for the reference sample DMSO (200 µl DMSO, 25 µl 0.2 M DMPO in DMSO, 25 µl 0.01 M $K_2S_2O_8$ in water) and for samples 8-OMTGM and isolated culture extracts E1–E2

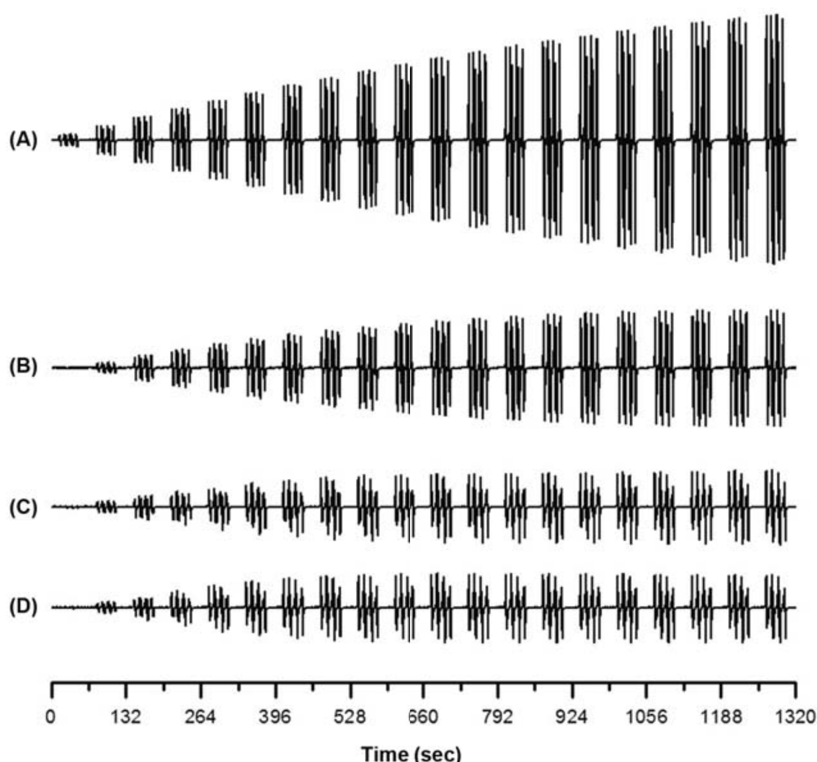

Fig. 6. **Sets of individual EPR spectra of DMPO spin adducts (magnetic field sweep 8 mT) observed in air-saturated solutions containing 25 µl 0.2 M DMPO in DMSO, 25 µl 0.01 M $K_2S_2O_8$ in H_2O and (A) 200 µl DMSO (reference system); (B) 200 µl of solution 8-OMTGM in DMSO (1 mg/ml); (C) extract isolated from *Streptomyces* sp. AC37 (E1), 200 µl of solution E1 in DMSO (1.955 mg/ml); and (D) extract isolated from AC37-9M-16 (E2), 200 µl of solution E2 in DMSO (1.5 mg/ml).**

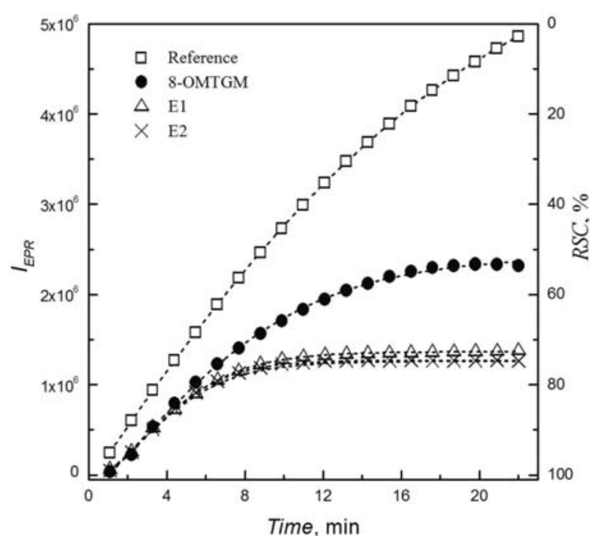

Fig. 7. Time course of EPR integral intensities (I_{EPR}) of DMPO spin adducts observed in air-saturated solutions for the reference system (□); and in the presence of 8-OMTGM (●); extract (E1) isolated from *Streptomyces* sp. AC37 (△); extract (E2) isolated from AC37-9M-16 (×). The data were obtained after double integration of EPR spectra shown in Fig. 6. Right axis illustrates the evaluation of Radical Scavenging Capacity (RSC) as "% of scavenged radicals" after 22 min incubation at 60°C.

(200 µl sample solution in DMSO, instead of DMSO in reference sample) is shown in Fig. 6. The EPR spectra measured in reference systems and in DMSO solution of 8-OMTGM were attributed to the spin adduct $^{\bullet}$DMPO-SO_4^- characterized by spin Hamiltonian parameters a_N=1.296 mT, a_H^{β}=0.938 mT, a_H^{γ}=0.139 mT; g=2.0059. However, in the presence of extracts E1–E2, formation of further DMPO spin adduct was observed, characterized by hyperfine coupling constants a_N=1.389 mT and a_H^{β}=1.176 mT. This spin adduct most likely may be attributed to the DMPO-adduct of oxygen-centered radical produced by the interaction of sample components with sulfate radical anions in the oxygenated DMSO solvent (Fig. 6).

After calculation of relative integral intensities of individual spectra, the EPR integral intensity after 22 min detected for the sample solution was compared to that of the reference (Fig. 7). The difference between these EPR intensities is proportional to the amount of radicals terminated by the scavengers present in the investigated sample. The RSC value of the sample is defined as a percentage of radicals scavenged relative to the reference sample representing DMSO solvent as shown in Fig. 7. The radical scavenging capacity of the sample evaluated in percent was recalculated to trolox equivalent antioxidant capacity using calibration curve measured analogously for trolox in $K_2S_2O_8$/DMPO/DMSO systems. Obtained RSC characteristics of investigated samples evaluated in mmol of trolox per 1 g of dry extract are summarized in Fig. 8A.

Antioxidant capacity determined by TEAC approach

Cation radical ABTS$^{\bullet+}$, prepared by oxidation of parent ABTS molecule with peroxydisulfate, represents paramagnetic species stable at room temperature (Re *et al.*, 1999). In the literature, this radical source is increasingly treated as a standard to characterize the antioxidant activity of various systems (Pellegrini *et al.*, 2003).

Solutions of 8-OMTGM and isolated extracts E1–E2 in DMSO revealed the ability to terminate ABTS$^{\bullet+}$ as shown in Fig. 9. The molar amount of ABTS$^{\bullet+}$ radical cation scavenged after 610 sec was used to evaluate antioxidant capacity of samples. The values of TEAC calculated from calibration curve constructed using trolox/ABTS$^{\bullet+}$ solutions that were in the range of 0.2–0.3 mM trolox per 1 g of dry sample (Fig. 8B), are lower than those found using the spin trapping technique (Fig. 8A). Different mechanisms are most likely involved in the scavenging of a stable ABTS$^{\bullet+}$ radical cation and the reactive $SO_4^{\bullet-}$ radical anion (Zalibera *et al.*, 2009) by active compounds present in the investigated samples.

Discussion

The *S. mediolani* sp. AC37-9M-16 produced metabolite identified as (-)-8-O-methyltetrangomycin according to LC/MS/MS spectra. The strain *Streptomyces* sp. AC37 has been mutated by mutagenic treatments and our results show that morphological mutants present alterations in (-)-8-O-methyltetrangomycin production. In our work, the initial screening was absolutely necessary to obtain better conditions for

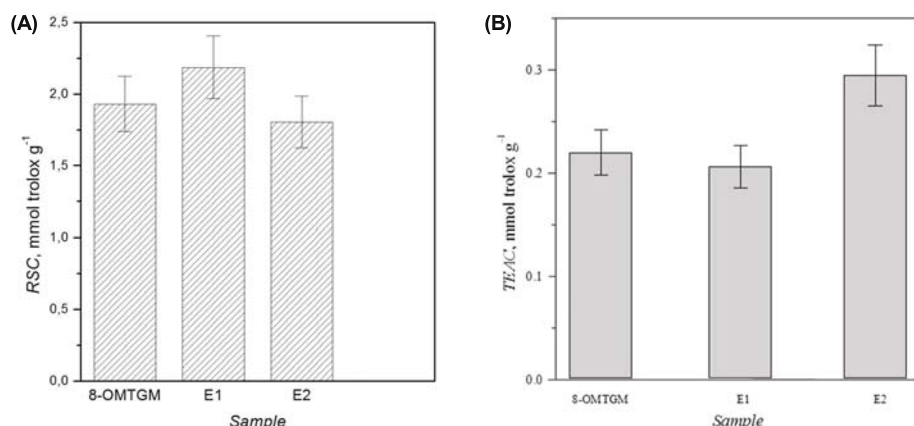

Fig. 8. (A) Radical Scavenging Capacity (*RSC*; expressed in mmol trolox per 1 g of dry sample) evaluated for investigated samples dissolved in DMSO employing the $K_2S_2O_8$/DMPO/DMSO assay at 60°C monitored by EPR spectroscopy (heating time 22 min). (B) Trolox equivalent antioxidant capacity (*TEAC*; expressed in mmol trolox per 1 g of dry sample) evaluated for investigated samples dissolved in DMSO using ABTS$^{\bullet+}$ approach with UV/vis detection (time after mixing 610 sec).

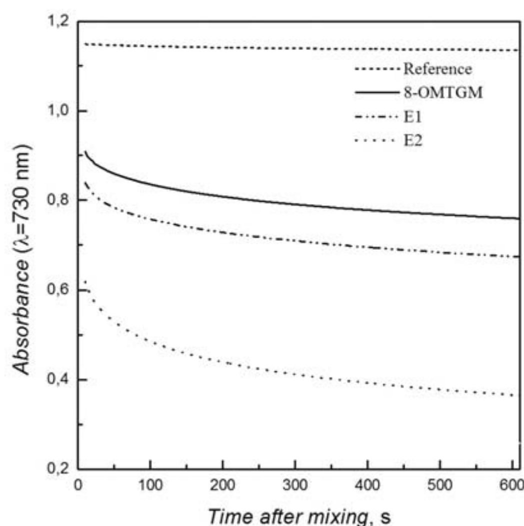

Fig. 9. Changes of ABTS$^{\bullet +}$ absorbance at 730 nm monitored after mixing of 200 μl of sample in DMSO with 2.8 ml of aqueous solutions of ABTS$^{\bullet +}$ (c_0=80 μM). In reference experiments the samples were replaced by DMSO solvent. Concentration of samples in DMSO solutions in mg/ml: 8-OMTGM: 1; E: 1.3; E2: 1.5.

finding the best 8-OMTGM producing mutants, the strain mutant AC-9M-16 exhibited the best producent. Morphological studies of the strain by means of electron microscopy revealed a clear pattern of its hyphal fragmentation into rod-shaped bacilli, a characteristic feature of the genus *Streptomycetaceae.* When comparing the electron microscopic images of *Streptomyces* sp. AC37 and mutant strains AC37-9M-16, substantial differences were revealed in morphology and ultrastructure. The major morphological change was larger inclusions in the mutant strains AC37-9M-16 compared to those in the original strain of *Streptomyces* sp. AC37. Due to the changes caused by mutation, the inclusions most probably grew in size.

The screening of new compound derivatives is closely accompanied by the study of auxiliary effectors for improved clinical results. Despite the vast selection of reported compounds, there is an ever-increasing need for new pharmacologically active molecules and potential drug leads. One of the standing goals is to find alternatives to overcome adverse clinical effects which currently compromise the use of many drugs in human therapeutics. This is classically exemplified by the immunosuppressor cyclosporine A used for reversal of multidrug resistance (Aouali *et al.*, 2005) and antioxidants applied to overcome anthracycline cardiotoxicity (Hideg and Kalai, 2007). In our study, we present two different treatments for the evaluation of (-)-8-O-methyltetrangomycin antioxidant capacity, not previously evaluated with a satisfactory outcome of the radical scavenging capacity, determined by EPR spectroscopy or antioxidant capacity determined by TEAC approach. This fact indicates that an induction by means of UV and NNMU influenced not only the morphology of the original *Streptomyces* sp. AC37, but also raised the capacity of angucycline production and new aglycone. We obtained mutants using NNMU as the genotoxic agent. These mutants might be useful for genetic studies and for the biosynthesis of (-)-8-O-methyltetrangomycin analogue producers in the future. Induced mutants can be achieved by means of different kinds of genotoxic agents. Marins *et al.* (1994), working with *S. aureofaciens,* obtained histidine-evertant mutants at high frequency with methyl methanesulphonate and N-methyl-N-nitro-N'-nitro-soguanidine (NTG) treatments. Considering the high content of C+G (cytosine+guanine) (about 74%) in the *Streptomyces* genome (Baltz, 1986) and the action mechanism of these genotoxic agents, they seem to be the most appropriate for mutation induction. Ikeda *et al.* (1988) used NTG and UV in *S. avermitilis* to obtain high avermectin producers and avermectin aglycone mutants.

The angucycline group metabolites have been shown to have a variety of biological activities, such as antibacterial and antitumor activity, and some act as inhibitors of oxidative enzymes. The suggested mode of action for the antitumor activity of this group is DNA alkylation, which is based primarily upon structure-activity relationships. For example, chemical modifications which lead to loss of the 5,6-double bond of various aquayamycin-type angucyclines results in breakdown of cytostatic activity (Rohr and Thiericke, 1992).

Therefore, in summary, an efficient method to enhance production of (-)-8-O-methyltetrangomycin includes the selection of mutagenic treatments using UV irradiation and chemical mutagenic treatment (N-nitroso-N-methyl-urea). All the combined parameters resulted in a considerable increase in (-)-8-O-methyltetrangomycin production. Future studies will be focused on optimization of production conditions (such as carbon and nitrogen supplement, temperature, pH and shaking). *Streptomyces* show commonly genetic instability, intra-strain morphological variability and co-relation between colony morphology and antibiotic activity (Schrempf, 1982). Although almost nothing is known about the mechanisms causing higher production in superior random or morphological mutants, it is likely that many of these mutations involve regulatory genes, especially as regulatory mutants obtained in basic genetic studies are sometimes found to be altered in colonial morphology. Thus, morphological mutants have been very important in strain improvement. These include mutants affected in mycelia formation, which produce colonies with a modified appearance or a new color (Adrio and Demain, 2006). The strain mutant AC37-9M-16 showed significant stability after one year.

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