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Research Article

Improvement of paromomycin production by *Streptomyces rimosus* subsp *paromomycinus* NRRL 2455 using gamma irradiation mutagenesis

Asmaa A. Ibrahim, Khaled M. Aboshanab*, Mahmoud A. Yassien, and Nadia A. Hassouna

Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt.

ABSTRACT

Streptomyces (S.) rimosus NRRL 2455 produces paromomycin, a 2-deoxystreptamine aminocyclitol aminoglycoside antibiotic (2DOS-ACAGA) with broad-spectrum activity against most of the Gram-positive and Gram-negative bacteria as well as protozoa. The mutation has become one of the beneficial methods used in enhancing the microbial production. Improvement of the paromomycin production by *S. rimosus* NRRL 2455 was achieved via irradiation mutagenesis using gamma (ɣ) radiation. The culture of *S. rimosus* was irradiated with different doses of gamma radiation (3, 4 and 5 KiloGray (KGy) to find out the best dose for the mutation that gave 99.99% killing. The optimum dose was found to be 4 KGy. Six morphologically changed colony types appeared on tryptic soy agar plates. These colonies were bio-assayed for their antimicrobial activity against standard *Staphylococcus aureus* ATCC 25923 using agar well diffusion technique. A mutant coded 5M showed about 1.44, and 2 fold increase in its activity as compared with the wild-type when cultivated in basal culture or optimized media (soybean meal 30 g/L, NH_4CL 4 g/L, $CaCO_3$ 5 g/L and glycerol 40 ml/L), respectively. Moreover, high genetic stability was observed upon subsequent culturing of 5M-mutant. Therefore, *S. rimosus* mutant-5M can be used as a potential industrial strain for paromomycin production.

Keywords: *Paromomycin, Streptomyces rimosus,* gamma mutagenesis, 2DOS-ACAGA.

***Correspondence** | Khaled M. Aboshanab; Professor of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, African union organization street Abassia, 11566, Cairo, Egypt. Email: aboshanab2012@pharma.asu.edu.eg

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1. INTRODUCTION

Streptomyces is a member of the family Actinomycetaceae. It is characterized by having mycelia and spores at the aerial hypha. They are attributed to the production of secondary metabolites such as antibiotics **[1]**. *S. rimosus* NRRL-2455 produces paromomycin, a 2 deoxystreptamine (2DOS)-containing aminoglycoside antibiotic with broad-spectrum activity against Gram-negative bacteria and most of the Gram-positive bacteria especially *Staphylococcus* strains particularly those resistant to oxytetracycline, erythromycin or carbomycin **[2,3]**. Members of *Streptomyces* display genetic instability, intra-strain morphological differences and there is also a correlation between colony morphology and antibiotic activity **[4]**. Random mutagenesis is considered as an effective way to improve the productivity of industrial microbial cultures **[5]**. It was found that some mutants with altered colony morphology and resulting from basic genetic studies exhibited enhanced activities **[6]**. Also, the change in the medium composition affects greatly the biosynthesis of the produced antibiotic from streptomycetes **[7]**. The current study aimed at enhancing the

paromomycin production from *S. rimosus* subsp. *paromomycinus* NRRL 2455 through gamma irradiation mutagenesis and showing the effect of the wild strain optimized media (unpublished data) on the hyper producer mutant.

2. MATERIALS AND METHODS

2.1. Microorganisms

S. rimosus subsp. *paromomycinus* NRRL 2455 was stored in tryptic soy broth (TSB) containing 50% glycerol at -20 °C **[8,9]** and cultured in TSB at 28 ˚C, 200 rpm and a 3-day incubation period for stock preparation to be used later for the preparation of a seed culture. *Staphylococcus aureus* ATCC 25923 was stored in Luria–Bertani broth (LB broth) **[10]** containing 20% glycerol at -20°C and cultured on nutrient agar slants at 37 ˚C for 24 h to be used in the bioassay of paromomycin production using agar well diffusion technique.

2.2. Culture Media

The basal medium for *S. rimosus* subsp. *paromomycinus* and the mutants were TSB **[9]**. *S. rimosus* optimized media was composed of soybean meal 30 g/L, NH_4CL 4g/L, $CaCO_3$ 5g/L and glycerol 40 ml/L.

2.3. Preparation of bacterial culture for paromomycin production

The seed culture was prepared by inoculating 25 ml basal medium in a 250 mL Erlenmeyer flask with 1.375 ml of the prepared stock and incubated for 72 h at 28 °C, 200 rpm. The main culture of *S. rimosus* was done in 25 mL TSB in 250 mL Erlenmeyer flasks at 28 °C, 200 rpm, and was then inoculated from the prepared seed culture.

2.4. Mutation Using Gamma Irradiation

After five days of incubation five mL aliquots from the above mentioned main culture (1.5×10^7 CFU/mL) were irradiated with different doses of gamma radiation (3, 4 and 5 KiloGray (KGy)) to find out the best dose for a mutation that gave 99.99% killing. The source of gamma radiation was ^{60}Co from Indian Gamma cell that was providing a dose rate of 2.2 KGy/h at the time of the experiment. The irradiation process was carried out at The National Center for Radiation Research and Technology, Atomic Energy Authority, Nasr City, Cairo, Egypt. After mutagenesis, the irradiated cell suspension was suitably diluted, plated on tryptic soy agar plates and incubated at 28 °C to get single colonies.

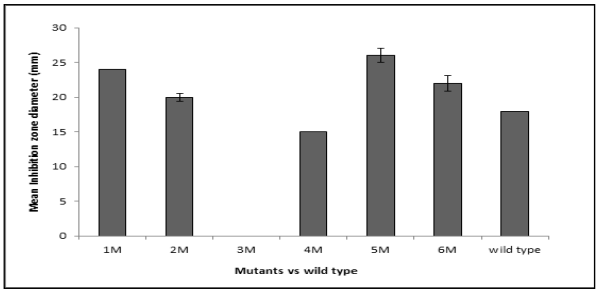
2.5. Determination of the antibacterial activity of the obtained mutants

To examine the antibacterial activity of the paromomycin produced by *S. rimosus* subsp. *paromomycinus* and its mutants, every single colony of the mutants and wild-type were conveyed to 25 mL of fresh TSB in a 250 mL Erlenmeyer flask and incubated for 3 days at 28 °C and 200 rpm. The obtained broths were used for the inoculation of the seed culture which was then used to inoculate the main culture as well as *S. rimosus* optimized media as the same procedure mentioned above. After each incubation process, the culture broths were centrifuged and sterilized by filtration using 0.22 µm pore size cellulose membrane filters (CHEM LAB, Barcelona, Spain). The culture filtrates were bio-assayed against *Staphylococcus aureus* ATCC 25923 by using agar well diffusion technique **[11]**. The suspension (0.5 McFarland) of *Staphylococcus aureus* ATCC-25923 was uniformly and aseptically spread on Mueller Hinton agar surface (MHA, Difco, USA) and 150 µl of the culture filtrate was used to fill the wells. Plates were kept at 4-8 °C for at least 30 min to allow the diffusion of culture filtrate. The diameters of inhibition zones were recorded after 24 h of incubation at 37 ˚C. All the experiments were done in triplicate and the mean of the three readings was documented.

3. RESULTS

The optimum dose of gamma radiation for the mutation that causes 99.99% killing was found to be 4 KGy. Gamma irradiation of wild-type strain caused the formation of six morphologically changed colony types, coded (1M, 2M, 3M, 4M, 5M, and 6M), on tryptic soy agar plates. These colonies were bio-assayed for their antimicrobial activity as shown in **Fig. 1**. All the mutants

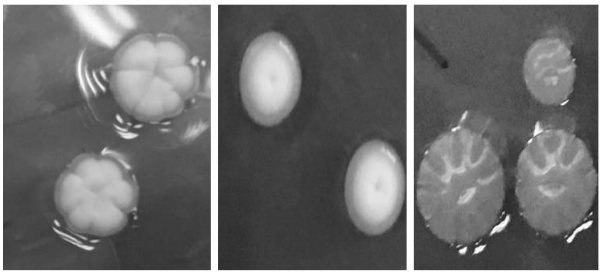
obtained showed higher antimicrobial activities than the wild type except for the two mutants 3M



and 4M.

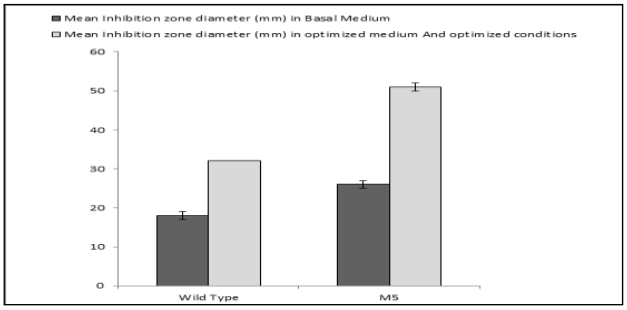
Fig. **1** Inhibition zone diameters of the six morphologically changed Mutants and Wild-type *S. rimosus* in the agar well diffusion assay.

The morphology of the mutant coded 3M colony showed the absence of the aerial mycelia as shown in **Fig. 2** and this morphological change was associated with loss of the antimicrobial



activity.

Fig. **2** The morphology of Mutants 5M and 3M compared to



wild-type on tryptic soy agar plates.

Fig. **3** Inhibition zone diameters of the wild-type and Mutant 5M after culturing in basal media and optimized media.

The best mutant was mutant 5M which showed about 1.44 and 2 fold increase in its activity as compared to the wild-type when cultivated in basal culture or optimized media, respectively as shown in **Fig. 3** Furthermore, successive culturing of 5M-mutant exhibited high genetic stability.

4. DISCUSSION

The mutation has been used for strain development to enhance the beneficial microbial secondary metabolites yield produced [5]and the screening for overproducing microbial mutants. These mutants' strains being overproducers offer the advantage of minimizing the production costs [6]. The most widely used mutagenic agents include methyl methanesulfonate (MMS), hydroxylamine (HA), ethyl methanesulfonate (EMS), N-methyl-N-nitro- N-Nitroso guanidine (MNNG) and ultraviolet (UV) irradiation **[12]**. The improvement of actinomycetes mutants has long been realized. The exposure of *Streptomyces* spores to ultraviolet and gamma rays develops hereditary changes that consequently cause colony morphology and pigmentation. These changes are greatly related to instabilities. These instabilities generate novel variants **[13]**. It was known that *Streptomyces* DNA contains high G+C content **[14]**. Furthermore, the best way to yield improvement is AT to CG transversions. So far, no AT to CG transversions has been reported by any chemical mutagen **[12]**. On the other hand, AT to CG transversions were found in cells treated with gamma rays **[15]**. Gamma rays induced mutation occurs through single or double-strand breakage of DNA resulting from deletion or structural change **[16]**. The application of gamma irradiation and the chosen dose depends on the types of biological materials where high doses are used for sterilization, medium doses for decontamination while low doses are used for mutagenesis **[17]**. In our study, *S.rimosus* subsp. *paromomycinus* was subjected to gamma rays producing six morphologically changed colonies. These colonies were bio-assayed against standard *Staphylococcus aureus* ATCC 25923. A mutant coded 5M showed about 1.44, and 2 fold increase in its activity as compared to the wild-type when cultivated in basal culture or optimized media respectively. Similar results were reported in previous studies. *Aspergillus niger*, a potent producer of many

important industrial enzymes, was genotypically improved by exposure to gamma rays **[18]**. *Aspergillus niger* producing α- and β-galactosidases enzymes revealed a two-fold increase in these two enzymes produced by its mutant after exposure to gamma radiation **[19]**. Also enhanced production of glucose oxidase was reported by mutants of *Aspergillus niger* after a dose of 80 krad **[18]**, while at a dose of 2 KGy, Mutant of *Aspergillus niger* improved its carboxymethylcellulose and filter paper cellulase production **[20]**. Moreover, Shahbazi et al. (2014) stated that a mutant strain of *Trichodermareesei* irradiated at 250 Gy of gamma ray (PTCC 5142) showed the maximum production of cellulose enzyme compared with UV-irradiated mutant and wild-type strains **[21]**. The tremendous increase was also reported with oxytetracycline production from a *Streptomyces rimosus* CN08 mutant which was called *Streptomyces rimosus* γ-45 **[22]**. Besides, a 12 fold increase was reported in the production of epsilon-pyrromycinone glycosides in *Streptomyces galilaeus* with respect to the wild strain after both natural selection and treatment with mutagens as gamma-irradiation **[23]**. Additionally in our findings, Successive culturing of the mutant 5M revealed great genetic stability. Similar results were reported for *Streptomyces fradiae* NRRL-2702 mutant gamma-1, however, UV irradiation associated changes were unstable with loss of tylosin activity **[24]**. These results make gamma radiation an effective way for mutation compared to other mutagens.

5. Conclusion

Genetic manipulation was carried out using gamma radiation and results revealed that gamma irradiation mutagenesis could generate production associated morphological changes in *S. rimosus* NRRL-2455. It was also noted that subsequent replating of the mutants showed same morphological changes and also constant paromomycin production upon successive culturing. A mutant coded 5M, the best mutant, showed about 1.44 increases in its activity as compared to the wild-type when cultivated in basal culture. It was interesting that culturing of mutant 5M in the *S. rimosus* subsp *paromomycinus* optimized media revealed a 2 fold increase in the production which is promising for scaling up the paromomycin production from this mutant strain. Therefore, Gamma Radiation was found to be very effective for improvement of paromomycin production and the obtained *S. rimosus* mutant-5M may be used as a potential industrial strain for paromomycin production in future studies.

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