Pharmacophore

ISSN-2229-5402

Journal home page: http://www.pharmacophorejournal.com

GENTAMICIN PRODUCTION BY CULTURAL OPTIMIZATION SYSTEM AND GENETICALLY IMPROVED MUTANTS OF *MICROMONOSPORA ECHINOSPORA*

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ARTICLE INFO

Received:
03 Jul 2019
Received in revised form:
14 Dec 2019
Accepted:
27 Dec 2019
Available online:
28 Feb 2020

Keywords: *Micromonospora echinospora* NRRL B-2995, gentamicin, EMS-mutants, optimization, RAPD-assay, phylogenetic-relationship

ABSTRACT

Ethyl methane sulfonate (EMS, 200 mM) mutagen was used to obtain superior producing gentamicin mutants *Micromonospora echinospora* NRRL B-2995 resistant to stress (1000 µg/ml of gentamicin). From 39 mutants, M40-12 was the highest gentamicin producing mutant. The results showed that this mutant gave 25 mm of zone inhibition by using *B. subtilis* as the tester strain with 178.57% in comparison with the parental strain. The 25 mm inhibition zone of M40-12 mutant equals 1500 µg/mL according to the standard curve of gentamicin; while, parental strain produced 800 µg/mL of gentamicin. So, the gentamicin improvement after EMS-mutagenesis was reached to 1.9 folds. Furthermore, the polymerase chain reaction (PCR) apparatus was used to detect the variabilities between superior gentamicin mutants compared to the parental strain using four random primers. The results indicated different fingerprints using random amplified polymorphic DNA (RAPD) assay by PCR. Also, the phylogenetic analysis was applied to divide the mutants under study into clusters which could reflect the genetic diversity of the new superior gentamicin mutants. Finally, gentamicin optimization by M40-12 mutant and the parental strain demonstrated that the optimum process parameters were a temperature of 34°C, and a pH of 7.5 for gentamicin production by the M40-12 superior mutant. Also, among all the tested organic carbon and nitrogen sources, dextrin and soybean flour were found to be more suitable for high yield of gentamicin production.

To Cite This Article: Shaza Y. A. Qattan, Khattab A.A., (2020), "Gentamicin Production by Cultural Optimization System and Genetically Improved Mutants of *Micromonospora echinospora*", ***Pharmacophore, 11(1),*** *15-26.*

Introduction

Beside *Streptomyces* and *Nocardia*, the genus *Micromonospra* has attracted much attention source of various types of the promising antibiotics. *Micromonospora* species, *echinospora* and *purpurea*, produces by fermentation a family of aminocyclitol antibiotics called gentamicin [1, 2]. Gentamicin is a broad spectrum, basic, and water-soluble antibiotic, first reported by Weinstein et al. (1965) [1]. Among the clinically more important species of Gram-negative organisms responsive to gentamicin are *Proteus, Pseudomonas, Escherichia coli, Aerobacter, Klebsiella, Salmonella,* and *Shigella* [3, 4]. Also, gentamicin is highly active against Gram-negative bacteria and *Mycobacterium tuberculosis* [5]. Besides being applied as an antibacterial agent, the potential anti-viral properties of some gentamicin conjugates recently have been detected [6].

The microbial strain development in general is a key for increasing of an antibiotic production as power antimicrobial bio-agents. The development of microbial strains to isolate the desired mutants was done using a very effective mutagenic agent. The random mutagenesis with the chemical and physical mutagens is an effective approach to increase microbial productivity. The Ethyl methanesulfonate (EMS) mutagen produces random nucleotide substitution which leads to the various point mutations in the microbial genomes by guanine alkylation [7, 8]. Different authors as [9-16] increased the production of antibiotic from various *Streptomyces* species by genetic enhancement systems.

To detect the genetic relationships within different microbial isolates, RAPD- assay has been used to make the DNA-fingerprinting of the microbial strains. The genomic polymorphism by RAPD assay has been applied to compare variability

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between the different bacterial strains at the intraspecific and interspecific levels [17-20]. This work was generated on EMS-treatments with *M. echinospora* to create gentamicin over-producing mutants and molecular typing of the excellent mutants. The goal of the present study was to adopt EMS-treatments and cultural optimization system to enhance the productivity of the agent**.**

Materials and Methods:

Microbes used and culture conditions:

Micromonospora echinospora NRRL B-2995 was obtained from United States Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Illinois, USA and used for gentamicin production. *Bacillus subtilis* NRRL 543 was used as the tester strain for gentamicin assay. The seed medium consisted of 1% glucose, 0.2% beef extract, 0.2% yeast extract, 0.2% polypeptone and 0.1% $CaCO_3$. The pH of seed medium was adjusted to 7.3 before autoclaving [21]. Fermentation medium (g/L) was used for gentamicin production: starch, 10; Soybean meal, 5.0; $CaCO_3$, 4.0; K_2HPO_4, 1.0; $FeSO_4 \cdot 7H_2O$, 0.03; $CoCl_2$, 0.001; 0.6 NaCl and the pH should be 7.6 [22]. The fermentation was continued for 5 days with 2% (v/v) seed medium inoculums before gentamicin determination.

Bioassay of gentamicin:

B. subtilis NRRL 543 used as the tester on nutrient agar (NA) medium to estimate the gentamicin production of each strain after mutagenesis. The antibiotic productivity was examined for the individual strain after inoculation with the tested isolates in the broth production medium for 5 days. The incubated production medium flasks were examined for gentamicin content by a paper disk diffusion method. The standard curve was prepared with authentic gentamicin (Figure 1).

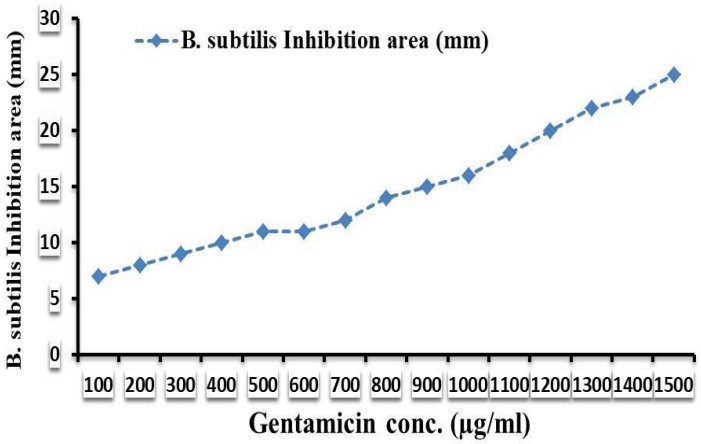

Figure 1: The effect of several concentrations of gentamicin on inhibition area with *B. subtilis* NRRL 543

Gentamicin-resistant mutants after EMS- mutagenesis:

The superior gentamicin-producing parental strain which was grown on seed medium for 4 days was mutated by EMS mutagen after centrifugation at 10000 rpm for 5 min of five ml *M. echinospora* spores and suspended in sodium phosphate buffer 0.1 M, pH 7. The bacterial spores were treated with a EMS mutagen (200 mM) for different periods, 20, 40 and 60 minutes and the treated spores suspension was then diluted and spread over the surface of the seed medium with and without gentamicin (1000 µg/ml) to select highly gentamicin resistant colonies. The plates are then incubated for 2 days at 30 °C. The colonies that developed with high growth rate were maintained on slants for another studies. To confirm their resistance, the resistant mutants with high growth rate were re-cultured on the same medium plates containing gentamicin for 2 days at 30°C.

Cultural optimization of gentamicin production:

Cultivation temperature: Fermentation was carried out at different temperatures (26, 28, 30, 32, 34 and 36°C) and gentamicin production was monitored as a function of temperature.

Initial medium pH: Growth was carried out in fermentation medium at different initial pH values (6, 6.5, 7, 7.5 and 8) and gentamicin production was monitored at the end of fermentation.

Carbon source: Growth was carried out as previously described utilizing the following carbon sources (1%, w/v): starch, dextrin, glucose, fructose, and sucrose.

Carbon source concentration: Six levels of dextrin (4, 6, 8, 10, 12 and 14g/l) were tested for their effect on gentamicin synthesis in the fermentation medium.

Nitrogen source: Organic sources (Soybean flour, peptone, tryptone, yeast extract, beef extract and meat extract) were evaluated for their effect on gentamicin synthesis. The medium used in this experiment contained (0.5%, w/v) different amounts of nitrogen sources.
Nitrogen source concentration: Four concentrations of soybean flour (3,5, 7 and 9 g/L) were tested for their effect on gentamicin synthesis.

Extraction of total DNA from the selected mutants and the wild type strain:
To isolate total DNA from the selected mutants and the wild type strain BYF DNA extraction i-genomic Mini Kit, iNtRON Biotechnology Inc., South Korea was used. The DNA quantity and purity was estimated as in [23] through reading the UV-absorbance at 260 and 280 nm using spectrophotometer (Shimadzu model UV-240).

Molecular variation of superior mutants by PCR-RAPD assay:
2xPCR Master Mix Solution (i-StarTaq), Hot-Start (iNtRON Biotechnology Inc., South Korea Product Catalog No: 25166) was applied for PCR analysis. The vials contain all of the necessary reagents, except primer and DNA template, for 25 µl total volume for in vitro DNA amplification. Four random primers were applied in the present investigation. The first primer (P2) sequence was 5′-CAT ACC CCC GCC GTT-3'. The second primer (P3) sequence was 5′-GTG TTG TGG TCC ACT-3'. The third primer (P4) sequence was 5′-AAC CTC CCC CTG ACC-3'. The fourth primer (P5) sequence was 5′-TGA GTG GTC TAC GTG-3'. All primers were supplied by Operon Technologies Company, Netherlands. The amplification protocol was carried out according to El-Sherbini and Khattab (2018). The amplified DNA products from RAPD analysis were electrophorated on 1.0% agarose gel and 1 X TBE buffer at consistent 100 volt for about 2 hrs. The sizes of the obtained different bands were confirmed against SiZer™ 1000 plus DNA ladder (iNtRON Biotechnology Inc., South Korea Product Catalog No: 24075) and the separated bands were stained with 10000X ViSafe Red Gel Stain (Vivantis# SD0103- Malaysia) and photographed using Gel Documentation System with UV Transeliminator.

PCR-RAPD data and phylogenetic assessment:
The obtained RAPD profiles for the three mutants were compared to each other as well as with the parental strain of *M. echinospora* NRRL B-2995. The bands of PCR-products were recorded as a binary data, where (1) means presence and (0) means absence of the band. To calculate phylogenetic relationships and genetic distance, the method of [24, 25] was used.

Results and Discussion:

Response of *M. echinospora*s NRRL B-2995 spores to EMS- mutagen:
The spore's solution of *M. echinospora* NRRL B-2995 was exposure to 200 mM of EMS-agent at several times (20, 40 and 60 min.) and then overlaid after several dilution on the seed plate medium. After EMS-mutagenesis, the appeared single colonies on the seed plates was checked on the seed plate supplemented with 1000 µg/ml of gentamicin by incubating them at 30ºC for 72 hrs. So, the colonies which able to grow on seed medium supplemented with 1000 µg/ml of gentamicin were registered as resistant mutants. It found an increase in the number of mutants with an increase in the treatment period up to 60 minutes (Table 1). On the other hand, the survival rate has reduced with the increase in the EMS-treatment period, i.e., it was 9.54% for 20 min. and highly reduced to reach 5.23 and 2.10% after 40 and 60 min of EMS-treatments, respectively. From about the 326 survival colonies obtained after EMS-treatment, 39 mutants showed a resistant mutant to 1000 µg/ml of gentamicin. The obtained results were exhibited that the survival ratio was reduced by elevating the EMS-treatment period. This reduction in survival ratio could be ascribed to the DNA damage and/or losses in other cell components caused by EMS-treatment. The obtained data were identical with those obtained by [15]. They observed the same result for survival rates after EMS-treatment of *S. lincolnensis*, since the less survival ratio was detected after the 60 min treatment time and increased after exposed to 40 and 20 min. of 200 mM EMS-mutagen.

Table 1: Survival of *M. echinospora* NRRL B-2995 colonies and gentamicin resistant after different exposure time of EMS mutagen

Exposure period (min)	Viable colonies No. and %		Resistant gentamicin mutants at 1000µg/ml	
	No.	%	No.	%
0	1834	100.00	0	00.00
20	175	9.54	17	9.71
40	96	5.23	13	13.54
60	55	2.10	9	16.36

EMS-mutagenesis and gentamicin productivity:
When the mutations induction using mutagenic agents interact with DNA, mainly the mutation produces a change in DNA nucleotide sequences. Mutations occur naturally are rarely compared to the mutations that induced, a DNA sequence changes such as base insertion, pair substitution, and deletion and are randomly occurred in microbial genome by

applications of physical and chemical mutagenic agents. To develop the bacterial genomes, the universal mutagenic sources such as ultraviolet light and alkylating agents (NTG and EMS), which commonly induce various mutations in the DNA of bacteria, are applied. EMS induces C-T variants resulting in C/G and T/A substitutions. Also, the 7-ethylguanidine produced by EMS hydrolysis results in G/C to C/G or G/C to T/A trans-versions; while, 3-ethyl adenine pairing errors cause A/T to G/C transitions [7, 8, 26]. The mutant strains are used to increase antibiotic production on a large scale and on an industrial level.

Evaluating of the 39-mutants was done by cultivating them in the fermentation medium for 5 days, to detect the effect of the EMS mutagenesis on gentamicin production. Table (2) exhibited that the parental strain gave 14 mm of zone inhibition by using the tester organism of *B. subtilis* as illustrated in Figure 2, only one mutant of them showed gentamicin at the same gentamicin production by the parental strain,16 mutants of them produced gentamicin more than the parental strain. The mutant strain E20-14 gave 24 mm of zone inhibition by using the tester organism of *B. subtilis* or 171.43% of the parental production and deemed as the best gentamicin producer after 20 min of EMS-treatment followed by the mutant strain E20-10 which gave 23 mm of zone inhibition by using the tester organism of *B. subtilis* or 164.29% of the parental strain as shown also in (Figure 2). It was found that the all tested mutants were resistant to 1000 µg/ml of gentamicin in comparison to the parental strain.

Moreover, when the thirteen mutants of gentamicin resistant which were obtained after EMS-mutagenesis for 40 min. for production of gentamicin (Table 3) were tested, T-40-5 mutant only produced gentamicin lower than that of the parental strain, 12 mutants out of 13 produced gentamicin more than the parental strain, three of them showed gentamicin produced up to 1.57 folds or more than that of the parental strain. The mutant strain M40-12 which gave 25 mm of zone inhibition by using the tester organism of *B. subtilis* or 178.57% of the parental production. It was the best gentamicin producer after 40 min. of EMS-treatment as in (Figure 2). The following best gentamicin producer after 40 min. of EMS-treatment was the mutant strain M40-10 which gave 23 mm of zone inhibition by using the tester organism of *B. subtilis* or 164.29% of the parental production. Furthermore, no any mutant produced gentamicin lower than parental strain after EMS-treatment for 60 min. (Table 4). The highest gentamicin producer after EMS-treatment for 60 min of was the S60-8 mutant that gave 24 mm of zone inhibition by using the tester organism of *B. subtilis* or 171.43% of the parental strain production as shown also in Figure 2. Finally, the following highest gentamicin producer after 40 min. of EMS-mutagenesis was the mutant S60-5 strain which gave 22 mm of zone inhibition by using the tester organism of *B. subtilis* or 157.14% of the parental production.

The same trends results were obtained by [27]. They exposed *Micromonospora echinospora* with different chemical mutagens like EtBr and MNNG and physical mutagens such as UV to obtain a mutant strain with gentamicin enhanced production. Also, Khattab (2011) used 250 µg/ml of Kanamycin as a selective pressure to isolate many of resistant mutants and produce higher kanamycin than their *S. kanamyceticus* wild type strain. Finally, Jin et al. (2002 a & b) reported improvement *A. mediterranei* rifamycin B production by rational screening after mutagenesis.

Table 2: Gentamicin production of the obtained mutants after treatment of *M. echinospora* NRRL B-2995 with 200 mM EMS for 20 min under 1000 µg/ml gentamicin stress

Code ofstrain	*B. subtilis* Inhibition area (mm)	% to Parental strain
Parentalstrain	14	100.00
E20-1	15	107.14
E20-2	17	121.43
E20-3	20	142.86
E20-4	16	114.29
E20-5	14	100.00
E20-6	21	150.00
E20-7	15	107.14
E20-8	16	114.29
E20-9	20	142.86
E20-10	23	164.29
E20-11	18	128.57
E20-12	15	107.14
E20-13	17	121.43
E20-14	24	171.43
E20-15	16	114.29
E20-16	18	128.57
E20-17	16	114.29

Table 3: Gentamicin production of the obtained mutants after treatment of *M. echinospora* NRRL B-2995 with 200 mM EMS for 40 min under 1000 µg/ml gentamicin stress

Code ofstrain	*B. subtilis* Inhibition area (mm)	% to Parental strain
Parental strain	14	100.00
M40-1	18	128.57
M40-2	15	107.14
M40-3	17	121.43
M40-4	18	128.57
M40-5	13	92.86
M40-6	16	114.29
M40-7	19	135.71
M40-8	22	157.14
M40-9	16	114.29
M40-10	23	164.29
M40-11	17	121.43
M40-12	25	178.57
M40-13	17	121.43

Table 4: Gentamicin production of the obtained mutants after treatment of *M. echinospora* NRRL B-2995 with 200 mM EMS for 60 min. under 1000 µg/ml gentamicin stress

Code ofstrain	*B. subtilis* Inhibition area (mm)	% to Parental strain
Parental strain	14	100.00
S60-1	17	121.43
S60-2	15	107.14
S60-3	18	128.57
S60-4	15	107.14
S60-5	22	157.14
S60-6	18	128.57
S60-7	20	142.86
S60-8	24	171.43
S60-9	18	128.57

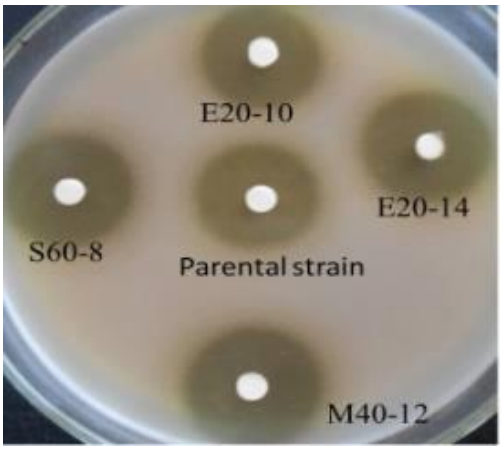

Figure 2: Gentamicin production of *M. echinospora* NRRL B-2995 (Parental strain) in comparison of superior mutants: E20-10, 20-14, M40-12 and S60-8

Gentamicin optimization of M40-12 mutant and parental strain:

Temperature degrees: Figure (3) depicts the effect of different incubation temperatures on gentamicin production. Temperature influenced gentamicin production and the optimum temperature to maximize gentamicin production for the parental strain was 32°C. On the other hand, the optimum temperature to maximize gentamicin production for M40-12 mutant was 34°C. Above or below this temperature a significant decrease in gentamicin production was noted.

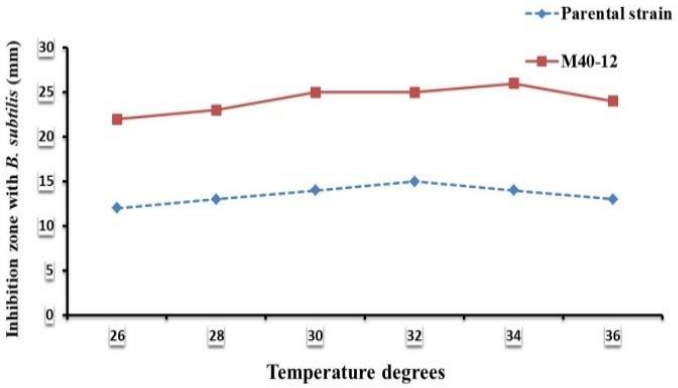

Figure 3: Gentamicin productivity using different temperature degrees

Different pH values: Figure (4) presents the effect of different pH values on gentamicin production. Gentamicin production showed a significant increase (15and 27 mm) for parental strain and M40-12 mutant, respectively, when pH value was increased from 7.0 to 7.5. Most of gentamicin producing fermentation is carried out between pH 6.0 and 8.0 and it varies from genus to genus [28].

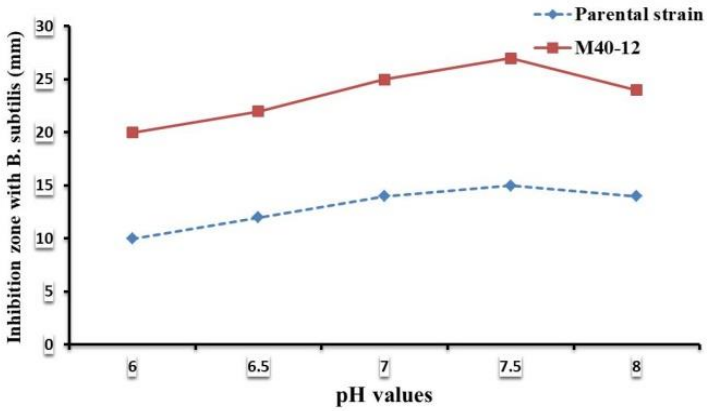

Figure 4: Gentamicin productivity using different pH values

Different carbon substrates: The carbon source is a critical substrate in the fermentation medium for the gentamicin production. Therefore, various carbon sources were tested for gentamicin production as shown in **Fig. 5**. Generally, carbon sources appeared to be more appropriate for the synthesis of gentamicin. Among all the tested carbon sources, dextrin was found to be more suitable for high yield of gentamicin production of parental strain followed by starch (15 and 14 mm, respectively). On the other hand, dextrin also was found to be more suitable for high yield of gentamicin production of M40-12 mutant followed by starch (26 and 25 mm, respectively). This result is an opposite with the observation of [22]**,** they reported that, among the various carbon sources, gentamicin production was the highest when starch was used followed by glucose.

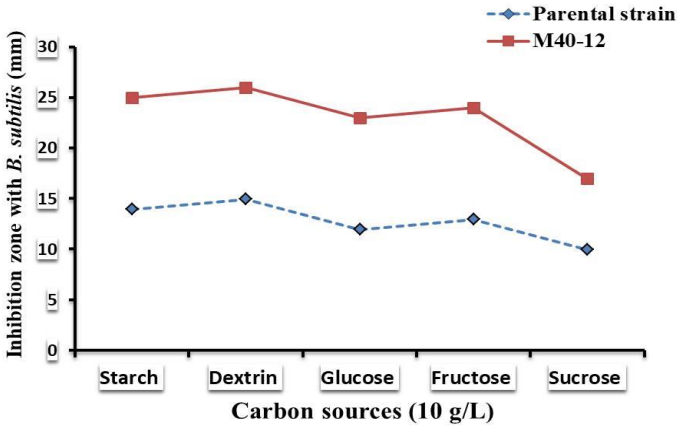

Figure 5: Gentamicin productivity using different carbon substrates

Different levels concentration of dextrin:

Different concentrations of dextrin (4 to 14 g/L) were tested for their effect on gentamicin production by parental strain and M40-12 mutant **(Fig. 6).** As dextrin concentration was increased, gentamicin production was increased as well and the 16 mm maximum productivity from parental strain and 27 mm maximum productivity from M40-12 mutant, were observed at 8 g/L dextrin, then the gentamicin production was decreased.

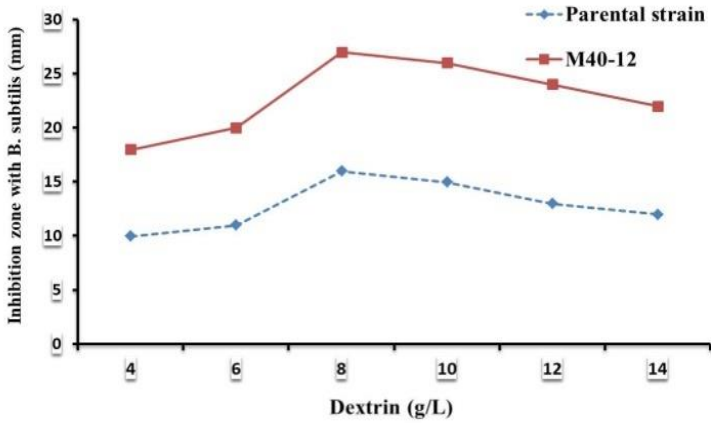

Figure 6: Gentamicin productivity using different concentration of dextrin

Different nitrogen sources:

Among all the tested organic nitrogen sources, soybean flour was found to be more suitable for high yield of gentamicin production of parental strain followed by tryptone (14and 13 mm, respectively). On the other hand, soybean flour was found to be more suitable for high yield of gentamicin production of M40-12 parental strain followed by tryptone (25 and 23 mm, respectively) as shown on **(Fig. 7).** A similar effect of nitrogen sources has been recorded for streptomycin [29] and neomycin biosynthesis [30]. Also, Himabindu and Jetty (2006) found that soybean meal was the most effective nitrogen source followed by sodium glutamate for gentamicin production by *M. echinospora*.

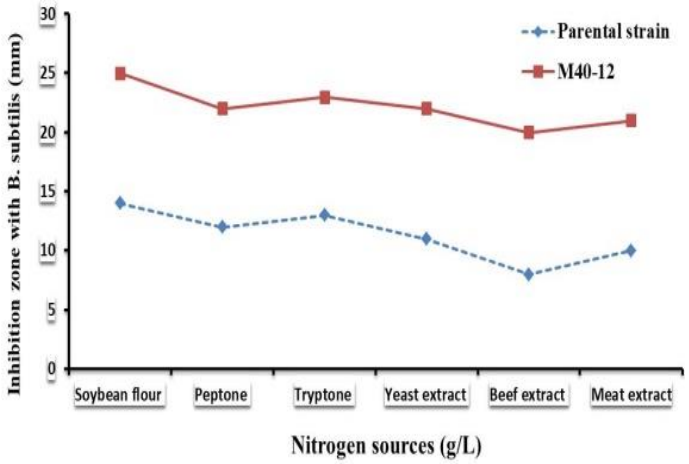

Figure 7: Gentamicin productivity using different nitrogen sources

Different concentration levels of soybean flour:

Different concentrations of soybean flour (3 to 9 g/L) were tested for their effect on gentamicin production by parental strain and M40-12 mutant (Fig. 8). As soybean flour concentration increased, gentamicin production increased as well and the 16 mm maximum productivity from parental strain and 28mm maximum productivity from M40-12 mutant were observed at 7 g/L soybean flour. This result is in agreement with the observation of **Himabindu et al. (2008)** who explained that the gentamicin production was increased significantly when the soybean meal concentration was increased.

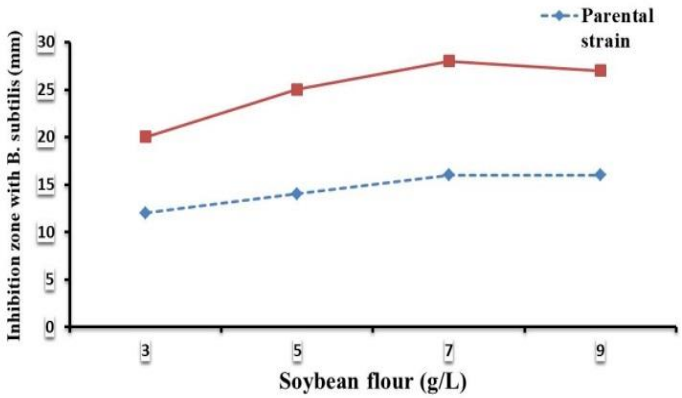

Figure 8: Gentamicin productivity using different levels of soybean flour

Molecular typing of the better gentamicin mutants:

Four RAPD-primers with three EMS-mutants as well as *M. echinospora* NRRL B-2995 (parental) were used to exhibit the molecular changes after EMS-treatments. The obtained banding patterns of the tested three EMS-mutants and parental strain when the random primer (P2) was applied as shown in Figure (2). One band (500 bp) was detected for the parental strain (lane 1). Also, the same distinct band (500 bp) was detected for the E20-10 mutant strain (lane 2) besides the very faint band (400 bp). The mutant strain E20-14 (lane 3) had the same band (500 bp) as the parental strain. Moreover, the mutant strain M40-12 (lane 4) had the same band (500 bp) as the parental strain and gave one new band (300 bp) compared to the parental strain.

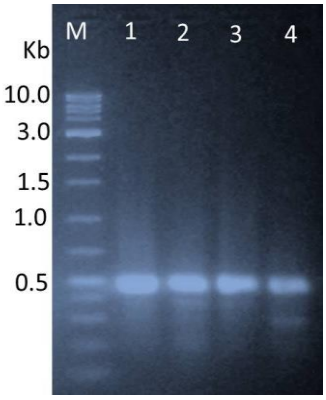

Figure 9: RAPD-DNA amplified banding profiles using primer (P2) for three tested mutants (Lanes 2 to 4) and *M. echinospora* NRRL B-2995 parental strain (lane 1) opposite to DNA Marker (lane M). The mutants sequence as follows: E20-10, E20-14 and M40-12

In using P3 primer (Figure 10) with the tested mutants in comparison of *M. echinospora* NRRL B-2995 (parental strain), one obvious band 400 bp was detected for the parental strain and in all of the tested mutants. The only exception was the mutant strain E20-10 (lane 2) which gave a distinctive and unique band (300 bp).

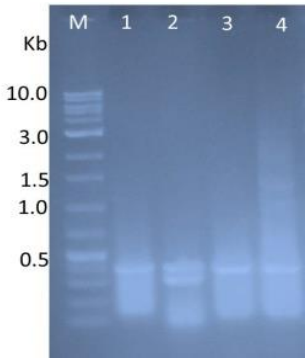

Figure 10: RAPD-DNA amplified banding profiles using primer (P3) for three tested mutants (Lanes 2 to 4) and *M. echinospora* NRRL B-2995 parental strain (lane 1) opposite to DNA Marker (lane M). The mutants sequence as follows: E20-10, E20-14 and M40-12

In the molecular description of the tested mutants by P4 primer compared to parental *M. echinospora* NRRL B-2995 strain (Figure 11), the parental strain (lane 1) gave one identical band of molecular weight (1250 bp). The mutant strain E20-10 (lane 3) exhibited an obvious unique band (1150 bp). The mutant strain E20-14 (lane 3) gave two unique bands (800 and 400 bp) besides the same band which was detected in the parental strain. The mutant strain M40-12 (lane 4) gave five unique bands (1000, 800, 700, 400 and 300 bp) besides the same band which was detected in the parental strain.
Finally, while using P5 primer (Figure 12) with the tested mutants in comparison of *M. echinospora* NRRL B-2995 *(*parental strain), three obvious bands (100, 600 and 500 bp) were detected for the parental strain and in all of the tested mutants. The only exception was that all tested mutants (lanes 2, 3 and 4) gave a distinctive and unique band (1100 bp).

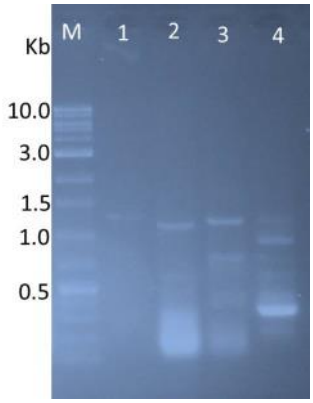

Figure 11: RAPD-DNA amplified banding profiles using primer (P4) for three tested mutants (Lanes 2 to 4) and *M. echinospora* NRRL B-2995 parental strain (lane 1) opposite to DNA Marker (lane M). The mutants sequence as follows: E20-10, E20-14 and M40-12

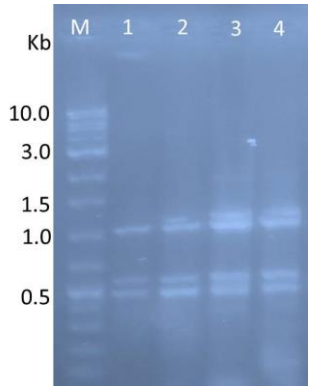

Figure 12: RAPD-DNA amplified banding profiles using primer (P5) for three tested mutants (Lanes 2 to 4) and *M. echinospora* NRRL B-2995 parental strain (lane 1) opposite to DNA Marker (lane M). The sequence of mutants was E20-10, E20-14 and M40-12 respectively

In general, the numbers of the DNA bands obtained after using RAPD-assay were recorded in (Table 5). When applied the primer P2 had 6 bands, primer P3 had 5 bands, and primer P4 had 11 bands. Also, 15 bands were obtained when the primer P5 was used. Furthermore, the highest polymorphic bands were obtained after using primer P4 and the less polymorphic bands were obtained after using the primer P5. No polymorphic bands were obtained with the primers P2 and P3. Moreover, the highest unique bands were exhibited with the primer P4 and the lowest unique bands were obtained after using primer P3. Two unique bands were produced when the primer P2 was used. Finally, when the primer P2 and primer P3 were used, the generated monomorphic bands were the same number (1 band) and no monomorphic bands were generated after using the primer P4. But, when the primer P5 was used, the highest monomorphic bands were detected.

Table 5: Number and types of the obtained DNA bands as well as the polymorphic percentages created by four RAPD-primers

Primer code	Total band No.	Monomorphic band No.	Unique band No.	Polymorphic band No.	Polymorphic percentage
P2	6	1	2	0	00.00%
P3	5	1	1	0	00.00%
P4	11	0	3	3	27. 27%

P5	15	3	0	1	6.67%
Total	37	5	6	4	-

The change in DNA synthesis will naturally have an impact on RAPD fingerprints. In theory, a single change rule could detect mutations in genomic DNA in this way. Modifications to the annealing sites of the primer may prevent the annealing and may result in the loss of the corresponding amplified band. Alternatively, new annealing sites can arise from mutations in the template, thereby producing new bands. There are several studies that use PCR-RAPD fingerprint protocols to distinguish between strains and mutants in bacteria [15, 31]. Moreover, it was assured that at the DNA level using RAPD-assay, not only the DNA of high mutant strains and the original strain were variable, but also the high number of different mutants' DNA were different.

Phylogenetic tree and RAPD assay:

To create the correct relationships at the level of the various genome sites, the genetic distances were performed on the obtained PCR-RAPD data as shown in (Table 6). The highest genetic distance percentage was detected between E20-14 and M40-12 (100%) followed by (75%) between parental strain from the side and E20-14, M40-12 from the other side. Finally, the less genetic distance matrix (63.20%) was detected in E20-10 and E20-14 and M40-12.

According the relationships as exhibited in (Figure 13), the dendrogram based on PCR-RAPD distance classified the mutants E20-14 and M40-12 into one first cluster and this cluster was connected to the parental strain in the second cluster. Finally, the highly different mutant E20-10 linked with the first and second clusters in the latest cluster.

The above cluster analysis was used to separate the studied mutants and the parental strain into categories which could be confirming the genetic diversities of the superior gentamicin producer mutants. Furthermore, the Figure (13) clarified that the novel genotypes (mutants E20-14 and M40-12) which assumed to be highly gentamicin producer was located at the same one cluster.

Table 6: Proximity matrix relationship within the superior mutants in relation to the parental strain based on RAPD assay

Strain code	Parental strain	E20-10	E20-14	M40-12
Parental strain	1.000	0. 667	0. 750	0. 750
E20-10	0. 667	1.000	0. 632	0. 632
E20-14	0. 750	0. 632	1.000	1.000
M40-12	0. 750	0. 632	1.000	1.000

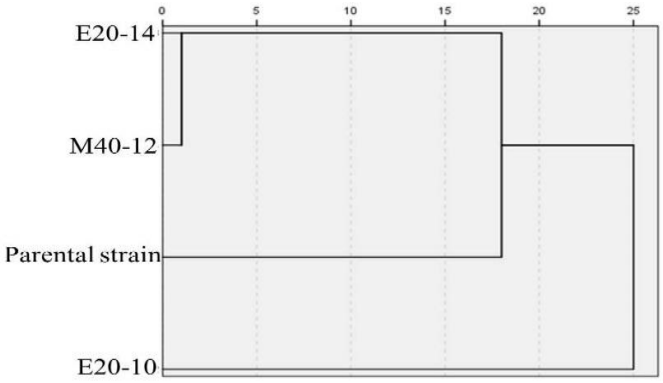

Figure 13: Phylogenetic tree through Average Linkage dendrogram of the three mutant strains against the parental strain based on RAPD protocol

The above results are in agreement with the results of different authors as [26, 31]. The parental and mutants strain *Lb. delbrueckii* NRRL B-1024 for ochratoxin biosorption were divided into the two main clusters according to RAPD-assay as reported by [26].

Conclusion

Our investigation has revealed that an enhancement of gentamicin productivity using some *M. echinospora* mutants after EMS-mutagenesis was produced. Furthermore, changes in RAPD patterns of some superior mutants in comparison with the parental strain were exhibited and these changes were proved at the same time with the evidence of genetic diversity of *M.*

echinospora superior mutants. The phylogenetic program was applied to divide the tested mutants into clusters which could reflect the high genetic variabilities of some superior gentamicin mutants. Finally, an improvement in gentamicin production was detected after optimization of the fermentation process.

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