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REGULAR ARTICLE

Multistage selection of soil actinomycete *Streptomyces albus* as a producer of antimicrobial substances

T. S. Todosiichuk[1], L. B. Zelena[2*] and V. V. Klochko[2]

[1]National Technical University of Ukraine «KPI», 37 Peremogy Pr., Kyiv, 03056, Ukraine
[2]Zabolotny Institute of Microbiology and Virology, National Academy of Science of Ukraine, 154 Zabolotnogo str., Kyiv, 03680, Ukraine

Abstract

Streptomycetes are known as the producers of various bioactive substances widely used in agriculture and many branches of industry. Different biotechnological methods and approaches are used to increased productivity of streptomycetes. In the present study, *S. albus* strains were subjected to mutagenesis associated with multistage selection and mutant strain with an increase of 3.5 times bacteriolytic enzyme complex production was obtained. Analysis of cell survival rate, antimicrobial and lytic activity as well as enzyme complex production in *Streptomyces* exposed to the various mutagen combinations (MNU, UV-irradiation, HNO_2) were carried out. Mutant strains were characterized by high bacteriolytic enzyme activity and significant antibiotic activity against *Kocuria* (*Micrococcus*) variants and *Candida albicans*. To estimate genomic changes caused by different mutagens, PCR with primers to short nucleotide repeats was performed and amplicons distinguishing parental and mutant strains were defined. The efficacy of the sequential mutagenic treatment for the hyper-producer obtaining as well as the relationship between the mutagen nature and the genomic variability has been shown. The results obtained in our research can be used and applied to the others *Streptomyces* species.

Key words: Enzyme complex, Lytic activity, Multistage selection, Nucleotide repeats, *Streptomyces albus*

Introduction

The traditional methods of microbial producers selection, such as mutagenesis and phenotypic selection, are still of current importance. They are applied both to choose bacterial hyper-producers and as the way of high biosynthetic level maintenance.

The last one is the most popular method which has been used for a long period of time to maintain the industrial microbial producers.

The current studies, in most cases, include multistage selection after sequential treatment of bacterial culture with several factors.

The most useful combination of affecting factors is the chemical and physical mutagens (Rasouli and Kulkarni, 1994; Matseliukh, 1998; Cheng et al., 2001; Liu et al., 2003). Usually, the effectiveness of applied scheme of microbial producer selection is defined as a value of culture productivity increasing that varied from 1.5 to several tens of times. However, in case of industrial perspective strains this index has to include production scale, the cost of culture media (substrates) and the final product. Sometimes, the increasing of product biosynthesis level in strain even by 1.5 – 2 times results in essential economic benefits when the strain has been implemented in industry. For example, the increasing biosynthesis of lipase enzyme not more than three times has been obtained in *Pseudomonas* and *Aspergillus* genus by applying method of multistage selection with various combinations of UV-radiation, nitrosoguanidine and HNO_2 (Caob and Zhanga, 2000; Ellaiah et al., 2002; Karanam and Medicherla, 2008). The same raise of bacteriolysin producing (up to three times) has been achieved in *Streptomyces recifensis* var. lyticus 2R 15 using methods of genetic engineering and the comparative analysis between peculiarities of transformants and parental strain showed significant differences (Babenko and Sokolova, 2009).

The majority of researches devoted to obtaining strains with an increase up to tens of times

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*Corresponding Author

L. B. Zelena
Zabolotny Institute of Microbiology and Virology, National Academy of Science of Ukraine, 154 Zabolotnogo str., Kyiv, 03680, Ukraine

Email: zelenalyubov@hotmail.com

bioactive substances productivity have studied mutant strains selected among wild environmental ones. Therefore, it is important to define regularities and direction of induced variability as well as influence of mutations on physiological characteristics regulated by polygenic system.

Microbial culture of *S. albus* (firstly identified as *S. recifensis var. lyticus*) is the producer of lytic enzymes complex and has been studied in a number of laboratories for some periods. The lytic enzymes complex comprises glycosidase, endopeptidases, muramidase and proteinases that together destroy microbial cells (Todosiichuk, 2000; Sokolova et al., 2004). Such specificity allows considering the enzymes complex as the basis of antiseptic medical and domestic means. The collection of strains possessing the increased substance activity and modified direction of biosynthesis has been obtained. The production engineering of the preparations of various purposes has been developed (Babenko et al., 2008; Pager, 2010). Various mutagens have been used separately to create these *S. albus* strains that were different in their productivity and product specificity.

In the present study, methods of multistage selection and mutagenesis were used to obtain *S. albus* strains with altered peculiarities and their genetic analysis was performed. The relationship between genetic and physiological characteristics of obtained strain-producers was analyzed. The possible rapid method of strain identification on the base of genome features was assessed. The determined regularities can be used to develop a scheme and procedures of selection other streptomycetes species – the most used industrial microbial cultures as producers of the various agriculture preparations (Postolachi, 2007; Anitha and Rabeeth, 2010; Hui et al., 2011) and enzymes for the food processes (Blattel et al., 2009; Syed et al., 2009).

Materials and Methods

Bacterial strains, media and culture conditions

S. albus strains 2435, 2435/M, US 101 were obtained from the collection of the Department of Industrial Biotechnology, National Technical University of Ukraine "Kyiv Polytechnic Institute". Strain 2435 was the parental strain and two mutants derived from it by different treatments: N-methyl-N-nitrosourea (2435/M) and UV-irradiation exposure (US 101). Mutant strains obtained in our study were designated as AE52 and UN44.

Streptomyces cultures were grown on the Petri plates with Chapek's agar medium at 28ºC for 7 days.

Mutagenesis

Treatment with N-methyl-N-nitrosourea (MNU)

5-days spore suspension was washed with 0.05 M phosphate buffer (pH 6.0) and incubated with N-methyl-N-nitrosourea (30 mg/mL) for 2 h in the Thermomixer comfort ("Eppendorf", Germany). To stop mutagen action spores were centrifuged at 12000 rpm for 10 min and twice washed with 0.05 M phosphate buffer (pH 6.0). Then suspension was cultured on the plates and cell survival rate was calculated.

UV-irradiation exposure

10 ml of spore suspension (1×10^8 cells/ml) in physiological solution was placed on Petri plate and subjected to UV-irradiation with constant agitation. The exposure was carried out by UV lamp with 254 – 255 nm wave length and 240 J/m^{2}dose. All manipulations were performed in the dark room to avoid photoreactivation. Cell survival rate was determined for every mutagen concentration.

Treatment with HNO_2 (nitrous acid)

0.2 ml of spore suspension (1×10^8 cells/ml) in physiological solution was mix with 5.6 ml of 0.2 M acetic buffer (pH 4.4 – 4.6) and 0.2 ml of $NaNO_2$ of various concentration (0.5, 1, 2, 3 M). 0.2 ml of physiological solution was added to control sample instead of mutagen. Samples were incubated for 10 – 80 min and then 0.5 ml was transfer to tubes with 4.5 ml of 0.2 M sterile phosphate buffer (pH 7.0) to stop mutagen action. The solution was cultured on the Petri plates with Chapek's agar medium and cell survival rate was counted. All individual colonies were used to measure their biosynthetic activity.

All mutant strains were analyzed to produce bacteriolytic enzyme complex as the selection criterion.

Estimation of biosynthetic activity

The strain biosynthetic activity was defined as lytic activity index (LAI) and calculated as the ratio of the diameter of the test culture lysis zone to the diameter of the entire colony. To determine LAI individual colony was transferred to the plates with medium consisting of (g/L): NaCl – 6.0, $K_2HPO_4\times3H_2O$ – 0.5, $FeSO_4$, $MgCl_2$, $MnCl_2$, $CuSO_4$, $ZnSO_4$ – each of 0.01; agar-agar – 25, pH 7.0; and *Escherichia coli* or *Staphylococcus aureus* as the test bacteria. The test bacteria culture was pre-heated at 90ºC for 30 min and added at concentration of $9x10^9$ cells/ ml.

The variation coefficient of culture variability was calculated as described (Pager, 2010).

The strain biosynthetic ability was characterized as percentage of "+"- and "-"-variants, i.e. clones with LAI differed from mean value by two standard deviations $|\bar{X}+2\sigma|$ and $|\bar{X}-2\sigma|$ respectively.

Biosynthesis of enzyme complex was carried out at 28±1°C for 5 days with agitation 220 min^{-1} in liquid medium (g/L): glucose – 6.0, soybean flour– 8.0, NaC –14.0, $CaCl_2$ – 4.5, $MgSO_4 \times 7H_2O$ – 5.8, $MnCl_2$ – 0.04, K_2HPO_4 – 1.5, H_2O – up to 1 L. The quantity of synthesized product (lytic activity) was measured with turbidimetric method (Pavlova et al., 1988).

Analysis of bacterial antagonistic activity

Bacterial antagonistic activity was analyzed according to Todosiichuk et al. (2014).

As reference strains were used: Gram-positive bacteria – *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 6538, *Kocuria* (*Micrococcus*) varians ATCC 9341; Gram-negative bacteria – *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 6896, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* NTCT 9027; yeasts – *Candida albicans* ATCC 10231, *Candida utilis* ATCC LIA-01. All strains were provided by Ukrainian Collection of Microorganisms, Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine.

Genetic analysis

Genomic DNA was isolated from cell culture using Proba-CTAB DNA isolation kit ("DNA-technology", Russia) according to manufacturer's instruction. The PCR mix composition and PCR program profiling are described in Kravets et al. (2012). For genome variability analysis primers 5'-$(GTG)_{5\text{-}3'}$ and 5'-$(GACA)_{4\text{-}3'}$ were used. The bands obtained after the amplification were recorded as present or absent in total amplicon pattern and binary matrix was constructed. Genetic distances were calculated on the basis of Nei-Li coefficient (1979) and the dendrogram was constructed with MEGA 5.0 program (UPGMA method).

Results and Discussion

Cell survival rate under various mutagens

Streptomycete strains, chosen for multistage selection were obtained by MNU (2435/M strain) and sequential treatment with MNU and UV-irradiation (US 101 strain) (Pager, 2010). Nitrite salt that generates nitric acid at low pH in water solution was used at the next stage of mutagenesis. Analysis of *S. albus* 2435/M and US 101 spore suspensions, subjected to $NaNO_2$ for 20 mins, revealed that at mutagen concentration as 0.5 M the strain survival rate was 47 – 50% while the increasing mutagen concentration (1, 2, 3 M) led to the enlargement of survival rate by 80 – 90%. The effect of various concentration of nitrous acid on cell survival rate was also observed in the other studies (Sidorkina et al., 1997; Petrea and Tofan, 2008).

The extension up to 40 min of treatment duration of *S. albus* 2435/M and US 101 strain spore suspensions with 0.5 M $NaNO_2$ allowed achieving 30 – 45% of cell survival and revealed variability of their sensitivity to the mutagen (Figure 1). *S. albus* US 101 had higher, than 2435/M strain, resistance and the survival rate of the other mutant subjected only to MNU was 30%. It is likely because bacterial UV-mutants possess photorepair that plays an important role in activation and functioning protective mechanisms in streptomycetes.

It is known that nitrous acid as mutagen is effective if the survival rate of microbial culture decreases by 10 – 50% (Sidorkina et al., 1997; Petrea and Tofan, 2008). The results, obtained in our research detected the higher level of resistance to this mutagen among *S. albus* strains; however, such indexes could be achieved at the treatment condition as follow: 50 min, 0.5 M $NaNO_2$.

Lytic activity of mutants

Analysis of *S. albus* mutant cells after treatment with $NaNO_2$ according to purposed scheme allowed us to determine the main influence regularities of mutagens used in the multistage selection (Table 1). A common pattern was a slight increase of the culture lytic activity variability (CV) against *S. aureus* (18.7 – 21.0%) and more significant against *E. coli* (12.7 – 20.2%). Since lytic enzymes complex that synthesized by culture comprised several compounds, the specific mutagen influence on general biosynthetic activity could alter the ratio of individual enzymes. It is possible that mutagen treatment could affect endopeptidases and proteinases activity, enzymes that break gram-negative bacterial multilayer cell wall. This fact is supported by the increasing LAI mean value against *E. coli* from 4.1 to 5.7, the maximal value of that was detected in culture seeding after three steps of mutagenesis. The percentage of culture "+"- and "-"-variants varied in the range of 1 – 2% among different mutants although they remained optimal concerning the II-staged mutants.

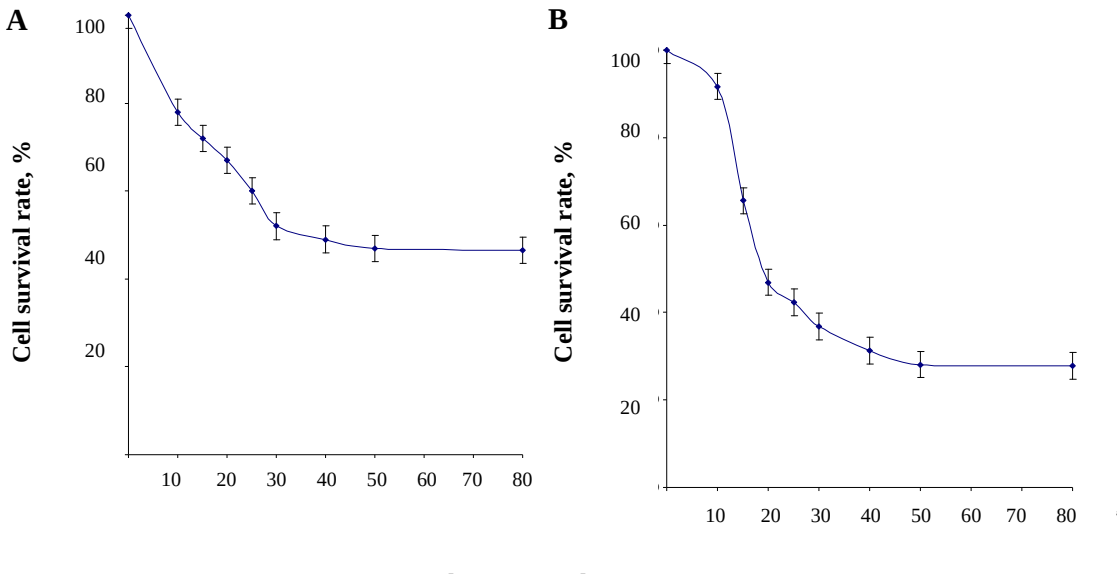

Figure 1. Cell survival rate of *S. albus* mutant strains under 0.5 M $NaNO_2$ treatment. (A) Strain 2435/M, (B) Strain US 101. Error bars indicate standard deviation between three replicates.

Table 1. Characteristics of *S. albus* strains after mutagenesis associated with multistage selection.

Characteristics	I stage mutagenesis* (2435/M)	II stage mutagenesis ** (US 101)	II stage mutagenesis *** (AE52)	III stage mutagenesis **** (UN44)
	E. coli/ S. aureus	*E. coli/ S. aureus*	*E. coli/ S. aureus*	*E. coli/ S. aureus*
Mean value of LAI, units	4.1/3.1	4.6/3.4	5.2/3.1	5.7/3.2
"+"-variants, %	3.3/5.2	4.1/4.8	1.7/5.4	2.5/3.3
"-"-variants, %	0.8/0	2.1/2.8	1.7/0	2.5/0.8
Variability coefficient (CV), %	13.5/18.7	12.7/20.3	20.2/19.0	16.0/21.0

Remarks: * - N-methyl-N-nitrosourea (MNU), ** - MNU + UV-irradiation, *** - MNU + HNO_2, **** - MNU + UV-irradiation + HNO_2

The highest positive impact on the increase of staphylolysin synthesis has been detected after treatment with a combination of chemical and physical mutagens (MNU and UV-irradiation). LAI mean value of US 101 strain seeding was 3.4.

Apparently, various mutagen treatments affected bacteriolysin synthesis in mutant strains that led to increasing of antimicrobial activity against gram-negative bacteria. Using of multistage selection allows choosing variants with positive changes accumulation. For example, the LAI mean value against *E. coli* depended on the mutagenesis stage: it was 4.1 in 2435/M strain (the I stage), 4.6 and 5.2 in US 101 and AE52, respectively (the II stage) and 5.7 in mutant strain of the III stage. Besides, seeding of the last one showed variants with LAI reaching up to 8, that was not defined among mutants of the previous stages. The variant UN44 that belonged to this group was used for the following analyses.

Evaluation of enzyme complex biosynthesis

To evaluate level of enzyme complex biosynthesis *S. albus* mutant strains, which were obtained by various treatments and different variants were cultured in liquid medium at mentioned conditions. Data presented at the Figure 2 showed that each successive stage of selection using different mutagens increased the biosynthetic activity of culture on average by twice.

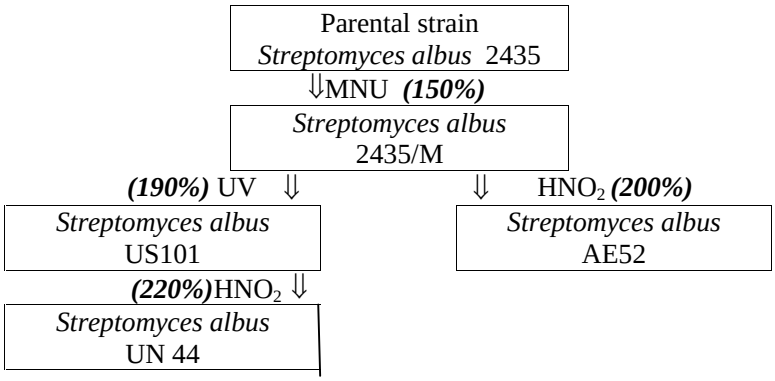

Figure 2. The scheme of multistage selection of *S. albus* and biosynthetic activity level (%) compared to previous variant.

In general, the proposed scheme of producer selection allowed obtaining strain with increased by 3.5 times bacteriolysin synthesis compared to parental strain. These data indicated the mean values of increased biosynthesis enzyme complex that could destroy both gram-positive and gram-negative bacteria. Thus, enlargement of both types of bacteria lytic activity against gram-positive and gram-negative bacteria was revealed. It is probably such way because the selected strains belonged to "+"-variants with the highest LAI value from 7 to 8.

To date the biosynthetic activity of 2435/M mutant strain exceeded the parental strain by 1.5 times while it was 2 – 2.5 times in 1998 when the strain was obtained (Todosiichuk et al., 1998). Partial loss of strain biosynthetic activity caused by the culture heterogeneity and variability (both natural and induced) testifies about the need of maintenance selection of producer including the use of different combinations of mutagens.

Antimicrobial activity of *S. albus* strains

Analysis of the literature on various *Streptomyces* as the producers of various biologically active compounds showed that their antimicrobial activity was often associated with synthesis of not one type of compounds but several of different nature. Therefore, the antibiotic activity was determined in the cultural liquid after biosynthesis and biomass removing at the previous stages of studying *S. albus* culture. However, unlike bacteriolytic action of the enzyme complex, antagonistic activity in the samples was not revealed. Thus, in the present study we analyzed the possibility of obtaining mutants with antibiotic activity using traditional test system for the antagonistic activity detection of the culture (rather than exogenous product as it was before).

The high antagonistic activity of *S. albus* parental and mutant strains was revealed against *Kocuria* (Micrococcus) variants and *Candida albicans*, no activity was revealed against the other analyzed gram-negative and gram-positive bacterial test cultures. Obviously, there was no correlation between the bacteriolysin over synthesis revealed in mutant strains 2435/M and UN44 and increased synthesis of antibiotic substances, but process of bacteriolysin synthesis was affected by mutagens used. The last observation might provoke chasing a strain-producer of antifungal substance, the variety of such strains are rather limited (van Wezel et al., 2009; Todosiichuk et al., 2011).

The lack of *S. albus* antagonistic activity against gram-negative and majority of gram-positive bacterial test cultures can be explained by the natural cell defensive mechanism. So, one of the products of metabolism (exogenous enzyme complex) targets the bacteria mainly and the other (endogenous antibiotic) has fungicidal action. The determined specificity of the culture defines its additional benefits as an industrial producer because there can be a possibility to obtain two antimicrobial substances during the same manufacturing process using both biomass and liquid culture.

Genomic analysis of *S. albus* strains

Damaging effect of mutagenic factors can cause genome changes in *Streptomyces*, rearrangements of nucleotide sequences in particular (Birch et al., 1991). To assess the variability of *S. albus* genome PCR analysis was performed using DNA-markers to microsatellite repeats $(GTG)_5$ and $(GACA)_4$ (Figure 3).

The total amount of bands after amplification with $(GTG)_5$ primer counted 9 fragments ranging in size from 350 to 1000 bp (Figure 3a). The smallest number of bands, 4, was found in strain 2435/M, the highest - in the strain US 101. Comparative analysis of amplicon patterns in parental and mutant strains showed that each sample had its own unique set of

fragments. Common in all samples were defined the fragments of 400 and 600 bp; only parental and mutant strain 2435/M (it was obtained some time ago and lost elevated synthesis level partially) were distinguished by the 800-bp band. Besides, there were fragments specific for only one among five strains; they can be used in further studies of strain identification. The 1000-bp amplicon allowed distinguishing parental strain and mutant strains: the band was observed only in mutant strains. The strain 2435 / M was characterized by amplification product of 500 bp and 650-bp fragment was observed only in the mutant (AE52) obtained without UV-irradiation.

Analysis of the amplification products pattern obtained with primer $(GACA)_4$ showed that the total spectrum included 11 PCR-fragments and their size varied from 300 to 1500 bp (Figure 3b).

Each sample was characterized by a set of bands and strain-specific amplicons were detected. The fragments of 600 and 950 bp were revealed in the strain subjected to both chemical mutagens and UV-irradiation (UN 44), the fragment of 800 bp was detected only in the parental strain. The fragment of 300-bp was defined as a distinguishing band of all mutant strains from the parental one.

Based on the results the genetic distances were calculated and dendrogram of genetic relationships between *S. albus* parental and mutant strains was constructed (Figure 4). The studied strains were divided into three groups: the first group combined parental strain 2435 and mutant US 101, the second group included mutants that were subjected to chemical treatment only (2435/M and AE52) and separate subgroup formed strain UN 44, obtained by three-staged mutagenesis with various chemicals and UV-irradiation.

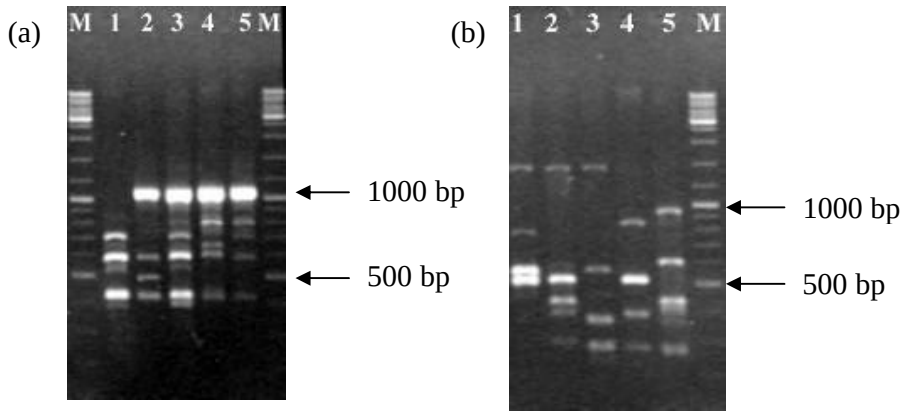

Figure 3. Electrophoregram of fragments of amplification with primers $(GTG)_5$ (a) and $(GACA)_4$ (b) and DNA isolated from *S. albus*: M – DNA ladder, 1 – 2435 (parental strain), 2-5 – mutant strains: 2435/M, US 101, AE52, UN 44, respectively.

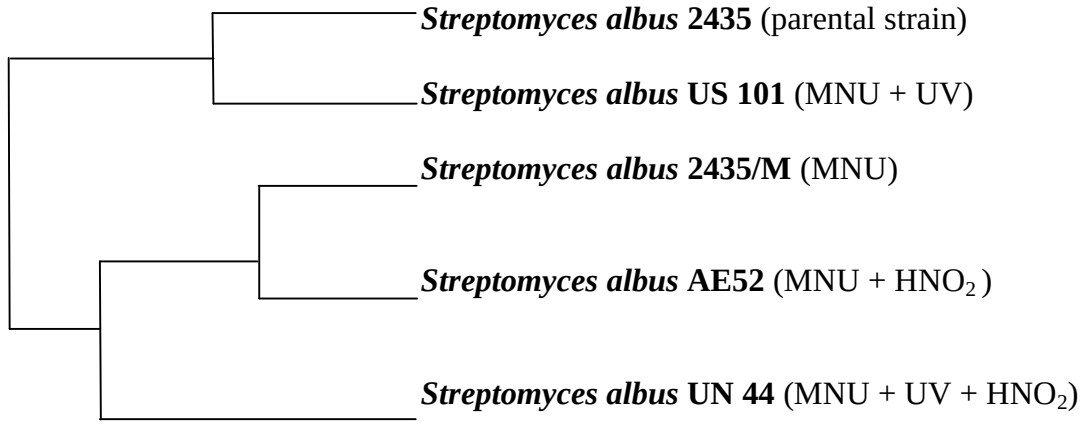

Figure 4. Dendrogram of genetic relationship between *S. albus* strains constructed with UPGMA method on the basis of PCR-analysis.

Distribution of strains between clusters suggested that the genome variability depended on type of stress factors, their combination and order of their application. Thus, the most distant from the parental culture was strain UN 44 and apparently minor DNA-changes caused by UV-irradiation in US 101 mutant led to its combining in one group with parental strain. Strain US 101 was originated from mutant 2435 / M, and hence from culture previously treated by chemical mutagens (see Figure 2). But the loss of the original activity, when strain 2435/M was stored for 15 years, obviously the result of a partial reversal events at the genetic level. That is why the second generation of UV-mutants (US 101) was genetically similar to the parental strain.

Conclusions

Thus, *S. albus* strain with increased up to 3.5 times bacteriolytic enzyme complex production was obtained as a result of mutagenesis associated with multistage selection carried out in the present study. The strain *S. albus* UN44 was deposited in Ukrainian collection of microorganisms as UCM IMV Ac-5030. The efficiency of multistage selection for maintaining culture activity and choosing new producer of bioactive substances was shown. It was suggested that short nucleotide repeats can be used for rapid identification of *S. albus* strains and for analyzing the specific effects of various mutagenic/stress factors on streptomycetes genome.

Author contributions

T. T design the study and conducted most of experiments: obtaining and selection of mutant strains, estimation of bacterial biosynthetic activity. L. Z. performed genetic analysis; V. K. was involved in analysis of bacterial antagonistic activity. T. T. and L. Z. prepared the manuscript and reviewed drafts of the paper. All authors read and approved the manuscript.

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