Increasing the productivity of hydrolase producers by induced mutagenesis and their comparative characteristics

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Abstract. In order to study the increase in productivity of natural hydrolase producers *B. subtilis* (producer of amylolytic and proteolytic enzymes) and *T. harzianum* (producer of xylolytic and cellulolytic enzymes), the efficiency of various approaches to non-directed induced mutagenesis was studied and their comparative characteristics were carried out. Using chemical and physical mutagenesis, new highly active producers of extracellular hydrolytic enzymes *B. subtilis* strain MB085-1457, *B. subtilis* strain MB085-2701, *B. subtilis* strain MB085-3689, *T. harzianum* strain MF091-962, *T. harzianum* strain MF091-2093 and *T. harzianum* strain MF091-3416 were obtained. Comparison of various methods of induced mutagenesis, namely methods of mutagenesis using nitrosoguanidine and nitrosomethylurea, UV and gamma irradiation showed that the greatest efficiency was achieved after mutagenesis using gamma rays. Various methods of induced mutagenesis can be used not only to create effective schemes for increasing the level of accumulation of target enzymes of producers, but also to obtain new drugs based on highly productive strains of microorganisms with hydrolase activity for the purpose of using them in resource-saving technologies for processing agricultural waste (into useful resources) and reducing the negative impact on the environment.
Keywords: mutant strains of microorganisms, producers, extracellular hydrolytic enzymes, induced mutagenesis, environmental protection

1 Introduction

The production of biologically active agents based on microorganisms and their enzymes is a leading direction in the development of modern biotechnology, and is one of the industries with a constantly growing volume of products and an expanding scope of use [1, 2]. Preparations of hydrolytic enzymes of microbial origin are widely used in the alcohol, confectionery, cheese-making, pulp and paper and pharmaceutical industries, including the

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production of biofuels, environmentally friendly washing powders, biocontrol of pollution of process flows and bioremediation of solid waste [3, 4]. A complex of hydrolytic enzymes (amylase, protease, cellulase, xylanase) together with microorganisms - producers of these substances are also used to replace expensive and environmentally hazardous chemicals, and to destroy various components of wastewater pollution [5, 6]. The specificity of action and high efficiency of microorganism enzymes contribute to obtaining target products with the required yield, which makes their use in industry economically feasible [1, 4].

The interest of researchers in microbial enzymes is due to the ever-increasing requirements for the safety of technological processes. The use of enzymes does not require huge expenditures on water and energy consumption, reduces emissions of carbon dioxide into the atmosphere, which is dangerous for the Earth, and the risk of negative changes in the environment [4, 6]. The practical significance determines the need to conduct research to improve existing and develop new methods for obtaining hydrolase enzyme preparations with new properties. First of all, enzymologists around the world are working to find microorganisms - producers of a highly active hydrolase complex that effectively destroy organic substrates and their derivatives.

In this regard, preference is given to bacterial and fungal strains capable of producing predominantly one extracellular enzyme or a group of enzymes with similar properties, in order to be able to use the latter to affect certain components of substrates [7, 8]. One of the current areas of research in this area is the production of new highly active mutant strains of microorganisms using mutagenesis methods [9, 10]. Among bacteria and microscopic fungi, the greatest interest as hydrolase producers is caused by representatives of the *Bacillus* and *Trichoderm*a genera due to a number of advantages over other microorganisms and the prospect of studying them for the possibility of using them in resource-saving technologies for processing agricultural waste (into useful resources) and reducing the negative impact on the environment [11, 12]. Previously, we screened hydrolytic enzyme producers [13], as a result of which strains of *B. subtilis* (a natural producer of amylolytic and proteolytic enzymes) and *T. harzianum* (a natural producer of xylolytic and cellulolytic enzymes) were discovered, and mutant strains differing in morphological and biochemical parameters were obtained by UV mutagenesis.

The aim of this work was to increase the productivity of hydrolase producers by induced mutagenesis and their comparative characteristics.

2 Materials and Methods

The following were used as objects of the study [13]: 1) *Bacillus subtilis* strain M085, a natural (original) producer of proteolytic and amylolytic enzymes; 2) *B. subtilis* strain MB085-148, a mutant producer of first-generation proteolytic and amylolytic enzymes obtained after short-term treatment with UV irradiation; 3) *B. subtilis* strain MB085-148-72, a mutant producer of first-generation proteolytic and amylolytic enzymes obtained after repeated treatment with UV irradiation followed by incubation in the dark; 4) *Trichoderma harzianum* strain MF091, a natural (original) producer of xylolytic and cellulolytic enzymes; 5) *T. harzianum* strain MF091-07 – mutant producer of first generation xylolytic and cellulolytic enzymes, obtained after short-term treatment with UV irradiation; 6) *T. harzianum* strain MF091-07-305 – mutant producer of first generation xylolytic and cellulolytic enzymes, obtained after multiple treatment with UV irradiation followed by incubation in the dark. Surface and submerged cultivation of strains was carried out using agar and liquid modified GRM and Sabouraud media containing exohydrolase substrates [13]. *B. subtilis* strains were cultured at 37-38°C for 2 days, *T. harzianum* strains – at 28-30°C for 7 days. The seed material (inoculum) was stored on a solid nutrient medium at 4 °C for no more than 2 months.

Induced mutagenesis of producers was carried out according to the following scheme [9, 14]: strain → treatment of suspensions of vegetative cells or spores with induced mutagen → plating of treated suspension on selective nutrient media containing substrates of target enzymes (with growth limiter) → counting percentage of surviving cells (number of CFU) → cultural, morphological and biochemical analysis of grown colonies → selection of producers by hydrolysis zones and transfer of selected variants to nutrient media → multiple testing of strains for stability by hydrolase activity during surface and submerged cultivation.

To enhance the activity of the target enzymes by chemical mutagenesis, 1 ml of a suspension of vegetative cells or spores (10^5-10^6 cells/ml) was treated with N-methyl-N-nitro-N-nitrosoguanidine or N-nitroso-N-methylurea at concentrations from 0.05 to 0.8%. The initial suspension of vegetative cells and spores was obtained by washing from the agar surface with sterile water and filtering through a glass filter with a pore size of 100 μm. The mixture of vegetative cells or spores with the mutagen was incubated for 0, 15, 30, 45 and 60 min at 30-32°C, after which it was washed three times with a phosphate buffer solution by centrifugation at 8000 g for 10 min. The suspension not treated with the mutagen served as a control. After incubation, five tenfold dilutions were prepared from each mutagen-treated suspension, which were cultured on selective nutrient media with substrates of the target enzymes [13, 14], and mutant variants exhibiting a high growth rate and hydrolase activity relative to the control were selected [13].

To carry out physical mutagenesis, a suspension of vegetative cells or spores (10^5-10^6 cells/ml) was irradiated with gamma rays with a dose of 300 to 3000 Gy. The suspension of vegetative cells or spores of microorganisms was in 5 ml Eppendorf microtubes. Gamma irradiation of the suspension was carried out at a temperature of 20-22°C for 0, 1, 2 h, 4 h, 8 h. The non-irradiated suspension served as a control. Irradiated vegetative cells and spores were seeded on selective media for qualitative and quantitative determination of the activity of exoenzymes [13, 14].

The efficiency of physical and chemical mutagenesis was analyzed by the degree of cell survival (number of CFU) of mutant variants [10] and their ability to produce exoenzymes in several generations [13]. The number of colony-forming units (CFU) was determined using the limiting dilution method [10]. The degree of cell survival of mutant variants (%) was analyzed as the ratio of the number of colonies grown in the experiment (after treatment with a mutagen) to the number of colonies in the control (without treatment with a mutagen), which was taken as 100%. Mutagenesis was considered effective if, after treatment with a mutagen, the resulting strain had an enzymatic activity of more than 10% compared to the activity of the parent strain.

The morphological and cultural characteristics of the strains were described on the second day of their growth for bacteria and the seventh day for fungi [14]. The cultural properties of the strains were studied by culturing them on solid and liquid nutrient media [15]. The morphological characteristics of the cells of the strains were studied by obtaining live and fixed stained preparations using light-field and phase-contrast microscopy.

Qualitative and quantitative determination of exoenzyme activity was performed according to the methods described by us earlier [13]. Qualitative determination of enzyme activity was assessed by the plate method. Quantitative determination of enzyme activity was analyzed in terms of milligram of protein that entered into the reaction. Protein concentration was determined spectrophotometrically by optical density values at 260 and 280 nm [16].

Statistical analysis of data from three independent tests was performed using the spreadsheet package of Statistica 6.0 (StatSoft Inc, USA), Matlab 6.5 (Mathworks Inc, USA) and MS Excel 2013 (Microsoft, USA). The normality of distribution of the obtained results was tested using the Shapiro-Wilk criterion. Differences in groups by average values of a quantitative variable were determined using the one-way analysis of variance (ANOVA) method at a significance level of $p \leq 0.05$. For intergroup comparisons, Tukey's post hoc test

was used ($p \leq 0.05$). To assess the relationship between the survival of vegetative cells or spores of strains and the dose (concentration) of their treatment with a mutagen, Spearman's (nonparametric method) and Pearson's (parametric method) correlation coefficients were calculated.

3 Results and discussion

To obtain highly productive strains of microorganisms producing amylolytic, proteolytic, xylolytic and cellulolytic enzymes and to characterize them comparatively, various approaches to non-targeted induced mutagenesis were used: treatment with nitrosoguanidine and nitrosomethylurea, and gamma irradiation. Our previous studies demonstrated the effectiveness of methods of ultraviolet (UV)-induced mutagenesis in a fractional mode and short-term UV irradiation [13].

At the first stage, the efficiency of induced mutagenesis was determined using chemical agents nitrosoguanidine and nitrosomethylurea (Tables 1 and 2). In order to identify the optimal parameters of chemical mutagenesis of natural producers *B. subtilis* M085 (natural producer of proteolytic and amylolytic enzymes) and *T. harzianum* MF091, the cell suspension was treated with mutagens at various concentrations (natural producer of xylolytic and cellulolytic enzymes).

Table 1. Results of determination of enzyme activity of microorganism strains after treatment with nitrosoguanidine at different concentrations*

Strain	Mutagen concentration, %	Enzyme activity, $d_{zones} / d_{colonies}$			
		protease	amylase	xylanase	cellulase
MB085	0	1.77±0.02*	1.61±0.02*	-	-
	0.05	1.81±0.03	1.67±0.03*	-	-
	0.1	1.84±0.04*	**1.79±0.02***	-	-
	0.2	**1.95±0.05***	1.72±0,04*	-	-
	0.4	**1.97±0.04***	1.68±0.03*	-	-
	0.8	1.88±0.03*	1.66±0.02*	-	-
MF091	0	-	-	1.68±0.02*	1.76±0.04*
	0.05	-	-	1.74±0.03*	1.86±0.04*
	0.1	-	-	1.71±0.03	1.85±0.03*
	0.2	-	-	**1.81±0.04**	**1.94±0,05***
	0.4	-	-	**1.77±0.04***	**1.99±0.06***
	0.8	-	-	1.69±0.03	1.87±0.05*
Note: * – Differences between options 0 and 0.05, 0.1, 0.2, 0.4, 0.8% are statistically significant at $p \leq 0.05$					

Table 2. Results of determination of enzymatic activity of microorganism strains after treatment with nitrosomethylurea at different concentrations*

Strain	Mutagen concentration, %	Enzyme activity, $d_{zones} / d_{colonies}$			
		protease	amylase	xylanase	cellulase
MB085	0	1.78±0.03*	1.62±0.03*	-	-
	0.05	**1.90±0.03***	1.68±0.02*	-	-
	0.1	1.85±0.02*	**1.75±0.03***	-	-
	0.2	1.81±0.03*	1.67±0.04*	-	-
	0.4	1.84±0.04*	1.58±0.03	-	-
	0.8	1.76±0.03*	1.64±0.04	-	-
MF091	0	-	-	1.65±0.02*	1.72±0.03*
	0.05	-	-	1.62±0.03	1.83±0.02*
	0.1	-	-	**1.78±0.03***	**1.91±0.05***
	0.2	-	-	1.70±0.03*	1.86±0.04*
	0.4	-	-	1.71±0.02*	1.78±0.03*
	0.8	-	-	1.66±0.03	1.74±0.03*
Note: * – Differences between options 0 and 0.05, 0.1, 0.2, 0.4, 0.8% are statistically significant at $p \leq 0.05$					

As a result of chemical mutagenesis, more than 2500 different mutant variants of the parent strains *B. subtilis* M085 and *T. harzianum* MF091 were obtained. Most of the microorganisms obtained by chemical mutagenesis either did not differ morphologically and biochemically from the parent strains, or the enzymatic activity of the mutants was lower than that of natural producers. Among the obtained variants, 4 mutants differed from the original strain in morphological and biochemical parameters. The enzymatic activity of these mutants, determined by a qualitative method, exceeded, on average, the activity of the original strains *B. subtilis* M085 and *T. harzianum* MF091 by 15-30%.

Since the efficiency of mutagenesis is assessed by the degree of microorganism cell survival, the most productive mutant variants were selected by determining the microbial count (CFU count). The most effective chemical mutagenesis of *B. subtilis* strain M085 and *T. harzianum* strain MF091 strains was caused by mutagen concentrations from 0.05 to 0.1%, at which the microorganism cell survival was from 3.4 to 5.1% (Figure 1, on the left). The value of the correlation coefficient between the cell survival of the strains and the concentration of chemical mutagens was more than 0.9, indicating a high (strong) correlation between these parameters (Figure 1, on the right). However, a negative relationship was found between these indicators, at which an increase in the concentration of chemical mutagens and a decrease in the survival parameter occurred.

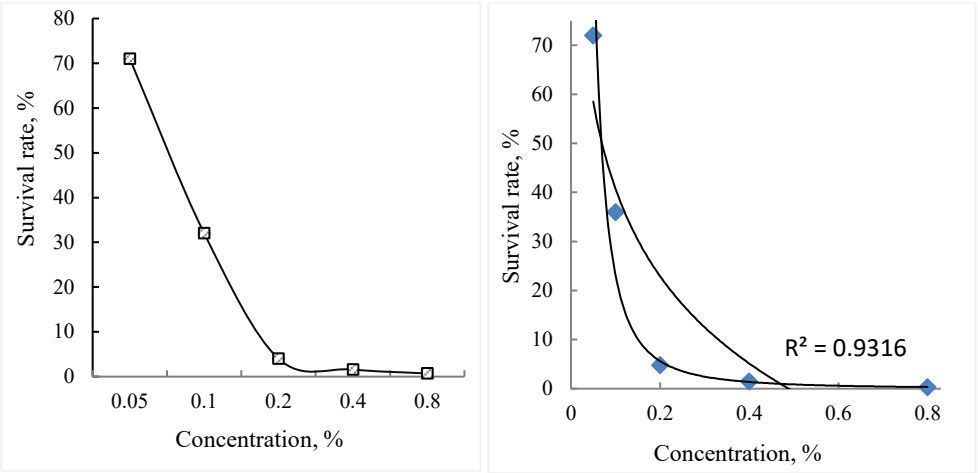

Fig. 1. Cell survival of the mutant variant of the *T. harzianum* strain (left) depending on the concentration of the chemical mutagen (nitrosoguanidine) during their treatment (right)

In order to study the efficiency of physical mutagenesis, gamma-irradiation of *B. subtilis* strain M085 and *T. harzianum* strain MF091 that had not undergone induced mutagenesis was carried out. Optimization of the gamma-irradiation parameters of the suspension of the original *B. subtilis* strain M085 and *T. harzianum* strain MF091 was carried out under different conditions (Table 3).

Table 3. Results of determination of enzymatic activity of microorganism strains after gamma irradiation under different modes

Strain	Radiation dose, Gr	Enzyme activity, d_{zones} / $d_{colonies}$			
		protease	amylase	xylanase	cellulase
MB085	0	1.75±0.02*	1.60±0.03*	-	-
	500	1.84±0.03*	1.68±0.04*	-	-
	1000	1.97±0.05*	1.72±0.02*	-	-
	1500	**2.18±0.06***	1.74±0.03*	-	-
	2000	1.92±0.03*	**1.81±0.03***	-	-
	2500	2.03±0.04*	1.71±0.02*	-	-
	3000	1.89±0.02*	1.66±0.02*	-	-
MF091	0	-	-	1.63±0.03*	1.71±0.03
	500	-	-	1.59±0.02	1.78±0.02*
	1000	-	-	1.72±0.03*	1.80±0.04*
	1500	-	-	1.83±0.04*	1.84±0.03*
	2000	-	-	**1.94±0.05***	**2.02±0.06***
	2500	-	-	1.77±0.04*	1.89±0.04*
	3000	-	-	1.68±0.04	1.75±0.03

Note: * - Differences between options 0 and 500, 1000, 1500, 2000, 2500, 3000 Gr are statistically significant at $p \leq 0.05$.

As a result of physical mutagenesis, more than 1250 different mutant variants of the wild *B. subtilis* strain M085 and *T. harzianum* strain MF091 were obtained. Treatment of the cell suspension with increasing doses of gamma irradiation was effective and provided an increase in the enzymatic activity of 3 strains by 35-57% at 1500 and 2000 Gy. Gamma mutagenesis at the indicated doses of irradiation provided the greatest decrease in survival (up to 0.06 to 1.1%) (Figere 2, on the left), which made it possible to select mutant strains with significantly increased enzymatic activity compared to the parent strains. In this case,

the survival of the cells of the mutant variants in the experiment varied depending on the dose of gamma irradiation, its duration, a negative correlation was observed between the survival of the cells of microorganisms and the dose of the mutagen (Figure 2, on the right).

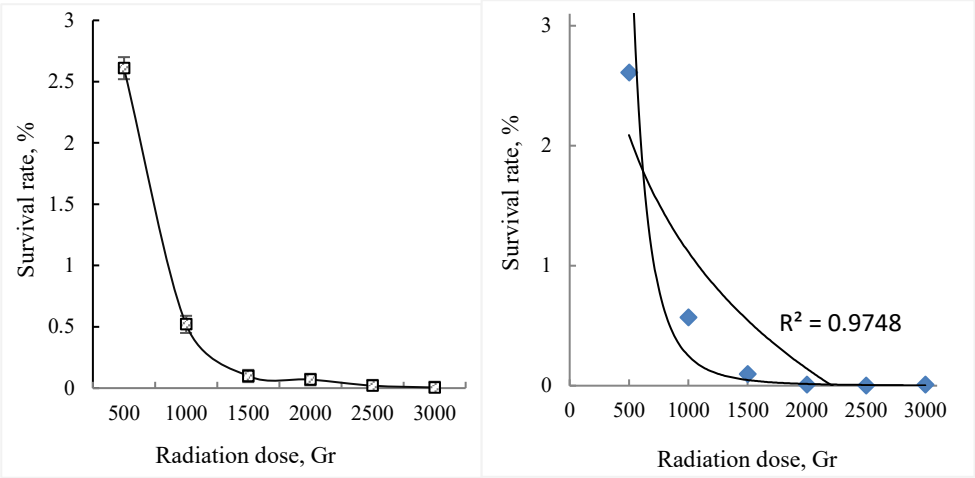

Fig. 2. Survival of spores of the mutant variant of the *T. harzianum* strain (left) depending on the dose of physical mutagen (gamma irradiation) during their treatment (right)

The results of the comparative evaluation of different approaches to non-targeted induced mutagenesis are presented in Table 4, in which the enzyme activity (%) is determined quantitatively.

Table 4. Results of a comparative study of enzyme activity of selected microorganism strains after using various induced mutagenesis approaches

Strain	Method of mutagenesis	Enzyme activity, % of control			
		protease	amylase	xylanase	cellulase
MB085-148	short-term UV irradiation	123.7±4.2	121.4±2.1	-	-
MB085-148-72	multiple UV irradiation + incubation in the dark	148.9±4.7	118.6±2.4	-	-
MB085-1457	gamma irradiation	159.3±6.0	141.9±5.8	-	-
MB085-2701	NG treatment	119.1±1.9	130.5±3.1	-	-
MB085-3689	NM treatment	116.8±2.3	124.0±3.6	-	-
MF091-07	short-term UV irradiation	-	-	117.2±3.8	112.7±1.7
MF091-07-305	multiple UV irradiation + incubation in the dark	-	-	131.6±4.5	138.4±3.9
MF091-962	gamma irradiation	-	-	154.8±6.1	146.0±5.3
MF091-2093	NG treatment	-	-	126.1±2.2	135.9±2.0
MF091-3416	NM treatment	-	-	122.2±2.4	118.5±1.6
Note: NG – nitrosoguanidine, NM – nitrosomethylurea					

It has been established that the use of agar nutrient media containing hydrolytic enzyme substrates is not always effective for the selection of some productive mutant variants. Literature data confirm our assumptions [10, 17] that the determination of highly active mutant strains by the hydrolysis zone of the corresponding enzyme substrates on agar nutrient media is not suitable for all microbial strains, since even productive variants can form small

hydrolysis zones. Therefore, the level of accumulation of hydrolytic enzymes by natural and mutant strains was determined in their culture liquid using a quantitative method.

A comparative analysis of various approaches to non-directed induced mutagenesis revealed that the most effective mutagenesis is the treatment of a microbial suspension with gamma rays. Induced mutagenesis using ultraviolet light [13], nitrosoguanidine, and nitrosomethylurea were less effective for the *B. subtilis* strain M085 and *T. harzianum* strain MF091. Along with this, when selecting mutant variants on selective nutrient media, fluctuations in the level of biosynthesis of exoenzymes of mutants were noted within wide limits and a decrease or absence of their hydrolase activity after several passages. The results obtained may be due to incorrect selection of the concentration (dose) of the mutagen or insufficient duration of its treatment. It should be noted that the discovered phenomenon can be explained by the presence of pre-replicative and post-replicative reparation systems in microorganisms, with the activation of photolyase and methylase enzymes in their cells [10, 14].

4 Conclusions

As a result of selection of the most effective method of non-directed induced mutagenesis, highly active mutant strains of *B. subtilis* MB085-1457, *B. subtilis* MB085-2701, *B. subtilis* MB085-3689, *T. harzianum* MF091-962, *T. harzianum* MF091-2093 and *T. harzianum* MF091-3416 were obtained. The most effective among chemical and physical mutagenesis of *B. subtilis* M085 and *T. harzianum* MF091 strains was gamma-ray treatment of microorganisms. The use of UV mutagenesis and chemical mutagenesis were less effective with respect to *B. subtilis* strain M085 and *T. harzianum* strain MF091. Despite this, various approaches to induced mutagenesis can be used to obtain highly productive strains of microorganisms producing amylolytic, proteolytic, xylolytic and cellulolytic enzymes in order to develop an effective scheme for increasing the level of accumulation of the target enzymes of the producer.

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