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ANNA PIĄTKIEWICZ
HELENA OBERMAN

INFLUENCE OF UV-RAYS AND NITROSOGUANIDINE ON THE PROTEOLYTIC ACTIVITY OF *LACTOBACILLUS CASEI*

Department of Technology of Fermentation and Microbiology Technical University, Łódź

Key words: *Lactobacillus casei*, UV-rays, N-Methyl-N-nitroso-N-nitroguanidine, proteolytic activity.

A suspension of *Lactobacillus casei* cells was expossed to UV-rays and N-methyl-N-nitroso-N-nitroguanidine (NTG) under standard conditions. The UV and NTG surviving cells showed increased proteolytic activity by 20-42% as compared with the parent strain.

INTRODUCTION

Mesophilic homofermentative lactobacilli represented by *Lactobacillus casei* constitute beside streptococci a significant component of microflora appropriate for ripening hard cheeses such as: Edam, Cheddar and others [4, 12, 19, 22, 23]. They are introduced as components of cheese-making starters, but they can also grow spontaneously in various kinds of cheeses and in fermented milk beverages. Taking into account the available data this group is thought to be responsible for the structure and flavour, as well as for the changes occuring in the maturation of cheese mass.

Organoleptic features typical for a given variety of cheese are the result of the adequate degree and "direction" of degradation of proteins and fat under the influence of the preparation coagulating milk (rennet) and of the enzymes of bacteria added with the starter.

The investigations on accelerating the process of cheese maturation and on improving their quality include the application of proteolitically active strains of bacteria or of the appropiate enzymatic preparations. The physiological properties of strains expressed by the growth rate, acid-producing and proteolytic activity are important criteria for the selection of

strains for the production of active biomass which is to substitute the traditional cheese-making starters. This paper presents the results of investigations on finding mutants of *L. casei* of a higher proteolytic activity by applying such mutagenic factors as UV-irradiation and nitrosoguanidine.

EXPERIMENTAL

MATERIALS AND METHODS

Strain

Lactobacillus casei W-10 was supplied by the Dairy Institute in Warsaw. The strain was stored at +4° on skim milk and on an acetate medium [1]. Periodically it was transferred to a liquid medium and milk (every 10-14 days). The strain was activated by three passages every 24 hrs in the nutrient media.
Incubation temperature +28°C.

Media

The strain was cultivated on an Elliker [7] liquid medium. To estimate the survival ability and isolation of the colonies the above mentioned medium supplemented with 2% $CaCO_3$ and 1.5% agar and agar-milk was used. To estimate the acidifying and proteolytic ability reconstituted skim milk (10%, tyndalized at 95°C, 15 min.) was used. The following control parameters were determined:

— the density of biomass in liquid media-spectrophotometrically at a wavelength of 540 nm. The extinction data were referred to the standard curve of bacterial turbidity as depending on the quantity of dry mass, determined gravimetrically.

— number of viable cells (CFU) by plating on an agarized medium.

— the acidifying activity — in pasteurized skim milk by titration 24-72 hrs old culture.

— the volatile acidity by destilation method [9].

— sugar content was assayed zsing anthrone method [8].

— pH potentiometrically.

— the level of amino-groups — in culture after seven days of growth in milk by the ninhydrine method [5], following precipitation of milk proteins with 12% TCA.

— specific acidifying activity expressed in ml of lactic acid formed by 1CFU in logarithmic phase of growth i.e. after 10 hrs of cultivation.

— the intracellular proteolytic activity of cell-free crude enzyme extracts using casein and its fraction alpha, beta, kappa in buffer solution

as a substrate was determined by two methods: 1. tannin method [13] the results were expressed in μg of casein consumed by 100 μg of proteins present in the cell-free extract, 2. ninhydrine method — the result were given in μg of $N\text{-}NH_2$ liberated from casein during incubation.

CONDITIONS OF MUTAGENESIS

The cells from the end stage of the logarithmic phase, i.e. after 18 horus of cultivation were exposed to the action of mutagenic factors. The cells of bacteria multiplied in Elliker's medium, were centrifugated, twice washed; with 0.2M sterilized phosphate buffer (pH = 6.5) and the washed precipitate of cells was suspended in buffer standarizing the density of the suspension to 1 mg of s.m./ml which corresponded to the number of cells ranging from 7.17×10^8 to 1.05×10^9 in 1 ml. The irradiation of cells was performed in standard conditions for 15-150 seconds. The source of rays was a UV bacteriological lamp with a power output of 5.4×10^4 erg/s/10 ml = 5.44×10^3 J/s/10 ml. N-Methyl-N-nitroso-N-nitroguanidin was used with the concentration range from 50-500 μg/ml of suspension for 15-180 min. at 28°C.

After the appropriate time of action of the mutagenic factor the cells were again centrifugated, washed with phosphate buffer with pH 6.5 and placed in the initial volume of the buffer. Then the cells were inoculated into solid media. The mutagenic factor was used once or several times at a few days' intervals after 2 passages of surviving cells on the nutrient medium.

At the associated action of UV-rays and nitrosoguanidine the suspension of bacteria subjected preliminary to the action of nitrosoguanidine (at a dose of 500 μg/ml, for 60 and 90 min.) was centrifugated, washed with phosphate buffer and irrediated with UV rays.

SELECTION OF ACTIVE STRAINS AFTER MUTATION

The first step of selection of the active strains was carried out on a agar-milk medium and in Elliker medium supplemented with chalk. The colonies with a zone of decomposition of $CaCO_3$ were inoculated into pasteurized skim milk, where after 7 days of cultivation the degree of digestion of milk casein was determined by level of amino nitrogen. The most active strains characterised by the increased yield of $N\text{-}NH_2$-groups, were submitted to the third stage of selection which included the determination of activity and specificity of peptide hydrolases of the most active strains against casein and its alpha, beta and kappa fractions.

RESULTS AND DISCUSSION

PROTEOLYTIC ACTIVITY OF MUTAGENIZED POPULATION

In the suspension of cells subjected to the action of mutagenic factors a drop of the number of viable cells proportional to action time of the factor and its dose was observed (Fig. 1). UV rays caused a very high lethal effect. After 150 secs of action of UV rays the survival of cells decreased to 5×10^{-4}-4×10^{-5}%. A similar effect of UV was observed in the case of streptococci [10, 17].

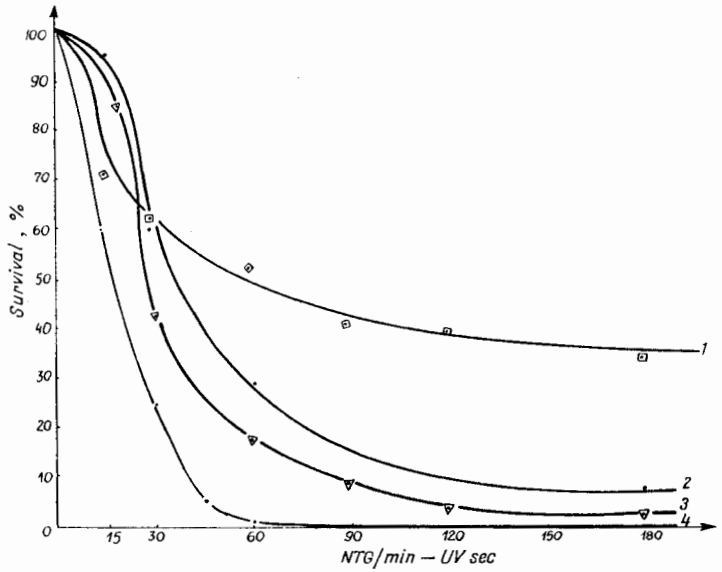

Fig. 1. Survival of *Lactobacillus casei* after: 1, 2, 3 — NTG treatment, 4 — UV treatment: 1 — NTG 50 μg/ml, 2 — NTG 300 μg/ml, 3 — NTG 500 μg/ml, 4 — UV-rays

Nitrosoguanidine caused a drop of the number of viable cells from 37.0% to 2.75% depending on concentration (Fig. 1). The repeated action of nitrosoguanidine induced an insignificant resistance of strains. The associated action of mutagenic factors increased the lethal effect and caused a drop of the number of viable cells to $2\text{-}3\times10^{-4}$%. Sub-populations recovered after mutagenesis did not reveal any morphological changes in relation to the parent population, independently of the mutagenic factor.

Proteolytic activity of mutagenized populations of *L. casei* was estimated for about 1000 populations which survived the action of mutagenic factors. They had a higher proteolytic activity i.e. an ability of decomposing milk proteins in comparison with the parent strain (Table 1). When examining the dispersion of this feature in the population for particular microorganism high heterogenity of proteolytic activity has

been found both in the parent population and in that subjected to the action of mutagenic factors. An increase of the homogeneity of that feature was obtained after the repeated action of mutagenic factor. The effect of UV rays increased as the radiation dose became higher. The most active UV-isolates represented 5% of the total number of clons coming from the population which was irradiated for 60 secs and 10% of the population which was irradiated for 90 secs. The increase of the radiation dose also eliminated from the population the proteolitically weakest forms. A similar effect and an increase of the number of the most active individuals were obtained after a twofold action of UV rays. A total of 16 UV subspecies. L. casei were isolated. Their proteolytic activity was increased from 20 to 40% in comparison with the parent strain.

Table 1. Proteolytic activity and survival of *Lactobacillus casei* recovered after UV and NTG treatment

Mutagenic agent	Survival (%)	mg % N-NH_2	
		range	average
Parent strain	—	6.50—7.75	6.94
UV—60 sec	1.3	6.30—10.0	7.07
UV—150 sec	4×10^{-5}	6.50—12.0	7.67
UV—60 sec twice	6×10^{-2}	6.50 10.0	7.68
Parent strain	—	22.4—32.4	27.2
NTG*	6.7	24.8—34.0	28.29
NTG*	7.7	28.8—35.6	32.24
NTG* twice	8.8	27.6—48.0	34.7
NTG*+UV**	$2.2—3\times10^{-4}$	20.0—39.6	31.2

* NTG treatment 90 min
** UV treatment 60 sec

Table 2. The effect of nitrosoguanidine (500 μg/ml) on the proteolytic activity of *L. casei*

Time of NTG treatment min	Range N-NH_2 mg%	Class of level of N-NH_2 (%)*)					
		I	II	III	IV	V	VI
30	11.4-26.0	—	6.7	53.3	26.6	6.7	6.7
60	14.4-33.8	—	—	6.7	26.6	46.7	20.0
90	16.6-36.4	—	—	—	13.4	40.0	46.0
120	12.0-30.8	—	—	13.4	33.3	40.0	13.3
180	13.8-29.0	—	—	33.3	26.7	20.0	20.0

*) Class I — to 15 mg% N-NH_2 V — 30-35 mg% N-NH_2
II — 15-20 mg% N-NH_2 VI — 35-40 mg% N-NH_2
III — 20-25 mg% N-NH_2 VII — 40-45 mg% N-NH_2
IV — 25-30 mg% N-NH_2 VIII — 45-50 mg% N-NH_2

A higher concentration of NTG/500 μg/ml/ and a longer time of its action — 3 min. (Table 2) revealted the most proteolitically active individuals. At that dose the greatest amount of active strains, about 46% was isolated. However the investigated population was heterogeneous and it showed considerable deviations in its ability of decomposing milk proteins.

Table 3. Proteolytic activity of *Lactobacillus casei* after associated NTG and UV treatments

Series*)	Mutagenic agent	Range N-NH_2 mg%	Class of level of N-NH_2**) %							
			I	II	III	IV	V	VI	VII	VIII
	Parent strain	22.4-32.4	—	—	10.5	73.3	15.8	—	—	—
I	NTG + UV	12.0-39.6	4.7	4.7	4.7	38.2	38.2	9.5	—	—
II	NTG + UV	24.4-35.2	—	—	5.7	22.9	57.2	14.2	—	—
III	NTG + UV	24.4-35.2	—	—	3.5	17.2	75.8	3.5	—	—
	Parent strain	23.2-30.8	—	—	16.0	76.0	8.0	—	—	—
IV	6 × NTG + UV	18.0-35.2	—	2.0	11.5	64.5	20.0	2.0	—	—
V	NTG + UV twice	27.6-48.8	—	—	—	15.0	65.0	12.5	5.0	2.5

*' Time of treatment UV-60 sec
Time of treatment NTG-90 min, series II-60 min

**' Explanation — table 2

The best results were obtained after twofold associated action of mutagenic factors UV and NTG. Such population showed an increase of proteolytic activity by about 48% and the level of aminoacid nitrogen in the culture of the most active mutant amounted to 48.8 mg% (Table 3). Singh et al. [21] observed a similar effect of nitrosoguanidine on the proteolytic activity of *Lactobacillus*. The applied mutagenic factors also increased homogenicity of *L. casei* populations as far as acid-producing ability was concerned eliminating weakly acidifying individuals. The isolates with

Table 4. The effect of NTG (500 μg/ml) on acidifying activity of *Lactobacillus casei*

Time of NTG treatment min.	Range of lactic acid g/100 ml of milk	Class of level of lactic acid*)		
Parent strain	0.615-0.974	13.3	86.7	—
15	0.662-1.022	13.3	80.0	6.7
30	0.738-0.974	—	100.0	—
90	0.588-1.160	20.0	40.0	40.
180	0.927-1.372	—	13.3	86.7

*' Level of lactic acid g/100 ml of milk
I — 0.4-0.7
II — 0.7-1.0
III — 1.0-1.3

Table. 5. Significance of differences in proteolytic activity of parent strain and mutagenized populations

Kind of population	Number of determination	Deviations of results N-NH_2 mg%	Mean result value	Standard deviation	Mean deviation	Range of reliability	Significance of difference		
							experimental value	theoterical value	
Parent strain	19	22.4-32.4	27.2	2.4	0.55	27.2±1.15	—	—	—
Parent strain	15	17.6-32.4	23.1	2.75	0.733	23.1±1.57	—	—	—
Parent strain	15	22.4-32.8	28.4	2.93	0.755	28.4±1.19	—	—	—
NTG twice	31	29.2-48.8	34.7	7.1	1.27	34.7±2.59	4.63	2.017	t t
NTG 500 g/ml	15	24.8-35.2	32.12	4.6	1.52	32.12±3.38	2.45	2.131	t t
NTG+UV	20	20.0-39.6	31.2	4.75	1.09	31.2±2.28	3.28	2.104	t t
Parent strain	20	6.40—7.75	6.94	0.392	0.087	6.94±0.18	—	—	—
UV 150 sec	77	6.50±12.25	6.67	1.29	0.15	7.67±0.29	2.48	1.98	t t
UV twice	75	6.50±10.0	7.68	1.07	0.12	7.68±0.29	2.99	1.98	t t
NTG 300 g/ml	23	3.20±20.0	10.63	2.75	0.58	10.53±1.59	3.74	2.086	t t

higher acidifying ability appeared after a longer time of the action of mutagenes (Table 4).

The isolated strains did not show any correlation between their acidifying and proteolytic activity. The calculated coefficients of correlation reveal its complete lack in the parent population ($r = 0.106$) and the existence of a very slight positive correlation ($r = 0.250$) in populations after the action of mutagenic factors. Many authors [3, 11, 25] as well as our previous data [17, 18] concerning streptococci do not show any correlation of these features in case of lactic acid bacteria, although Maksimowa [11], found agreement between acidifying and proteolytic activity for certain *Streprococcus lactis* and *Streptocossus diacetilactis* strains.

The significant differences between the average results of the particular series of experiments and the control tests calculated in a "Student's" test point to essential differences in the proteolytic activity of mutants obtained after the action of all mutagenic factors in relation to the parent strain (Table 5).

CHARACTERISTICS OF THE OBTAINED MUTANTS

Characteristics of mutants obtained in particular series of experiments included the estimation of their growth, acidifying activity as well as the specific proteolytic activity of cell-free extracts against isoelectric casein and its alpha, beta and kappa fractions. The dynamics of strains growth was similar and the maximum yield of biomass ranged from 0.7-1.1 g/l depending on the medium (Table 6). The end of the logarithmic phase occurred in the 18th-20th hour of cultivation. The mutants had higher acidifying ability and volatile acidity in comparison with the parent strain, e.g. the strain 5/3 caused curdling of milk after 14 hrs of culture. The greatest differentiation between the parent strain and mutants is proved by the investigations of specific proteolytic activity (Fig. 2). Most of the isolated strains digested the β-fraction and isoelectric casein more strongly than the parent strain. Strain 5/3 revealed higher activity against alpha fraction, whereas UV-mutant 60/8 against kappa casein and at the some time the level of aminoacid mitrogen increased in the medium, which gave evidence of the high ability of liberating aminoacids from that substrate.

These results are confirmed by the differentiation of the specific proteolytic activity of particular varieties of lactic acid bacteria against alpha and beta casein. Thus degradation of these two fractions in the process of theese ripening depends on the varietf of bacteria used in starters. Various authors [20, 24] observed a stronger degradation of alpha casein or beta casein [14, 15,.16].

The results of investigating the specificity of peptide hydrolases of *L. casei* mutants isolated in the present paper have thus confirmed in this case the usefulness of a preliminary selection of strains proteolitical-

Table. 6. Growth and activity of *L. casei* recovered after UV and NTG treatment

N°	Biomass yield g/l	Generation time	CFU/ml after 24 hrs cultivation		pH Elliker	Sugar utilization %	Lactic acid g/100 ml of milk after 72 hrs cultivation	Specific acidifying mM/CFU after 18 hrs $\times 10^{-10}$	Volatile acidity mg%	N-NH_2 mg% after 7 days cultivation in milk
			Elliker*	milk**						
Parent strain	0.620	4.6	0.99	0.47	4.4	83	1.11	9.1	4.5	25.2
UV 60/8	0.725	—	1.03	—	4.4	86	0.710***	—	—	10***
UV 90/39	0.715	—	0.86	—	3.8	81.3	0.740***	—	—	9.3***
NTG 5/3	1.0	2.7	1.5	1.0	4.47	71.7	1.59	2.04	6.0	31.3
NTG 90/8	1.04	5.2	1.5	0.72	4.7	80.2	1.16	4.2	5.5	39.4
NTG 60/7	1.03	4.8	3.3	0.92	4.7	75.3	1.21	4.1	3.9	37.6
NTG+UV/2	0.740	4.4	2.8	0.5	—	—	1.305	9.1	5.6	37.9
NTG+UV/10	0.710	3.9	2.3	0.51	—	—	1.243	8.9	5.3	37.9

*' Elliker medium
**' Milk medium
**' After 24 hrs cultivation of milk

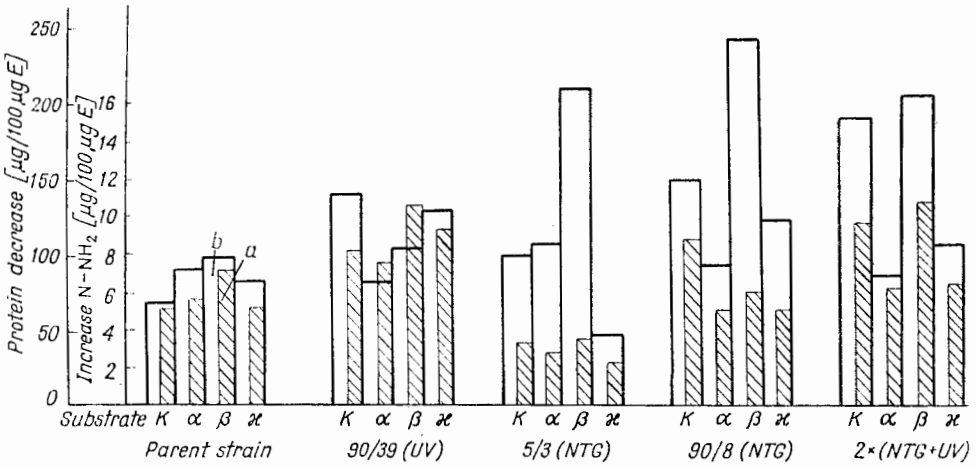

Fig. 2. The proteolytic activity of cell-free extracts (E) of parental and mutant strains of *L. casei*: a — protein decrease µg/100 µg (E), b — $N\text{-}NH_2$ increase µg/100 µg (E)

ly active on the basis of the level of aminoacid nitrogen in culture and in milk. The isolated strains with the increased and differentiated abilities of attacking various casein fractions may be useful in the dairy industry, giving products of good quality and quickening the ripening process of cheeses [2, 6].

CONCLUSIONS

— The investigations carried out by us and the suggested sequence of selection allowed to abtain proteolitically active strains of lactobacilli.

— Proteolytic activity is a heterogenous feature in the population of these bacteria. Mutagenic factors of the UV type and nitrosoguanidine eliminated the weakest individuals from the population, their proteolytic activity being correlated with the radiation dose and concentration of the chemical agent.

— The mutagenic factors applied also eliminated from the population the individuals with weak acidifying ability, but there was no correlation between those two physiological features.

— Significant differences have been found in the proteolytic activity of the parent strain and the mutants obtained after the action of all the mutagenic factors applied.

LITERATURE

1. Barton-Wright E. C.: The Microbiological Assay of the Vitamin B-complex and Aminoacids. New York. Pitman Publ. Corpor., 1952, 54.
2. Bannikova L. A., Maksimova A. K.: Mol. Prom., 1969, 3, 15.

3. Bottazzi V.: Il potere proteolitic dei lactobacilli e il processe di maturaziome dei formaggi. Il Latte 1961, **8**, 597.
4. Branen A. L., Keenan T. W.: J. Microbiol., 1970, **16**, 947.
5. Colovick P., Kaplan Nathan O.: Methods in Enzymology. E. Sidney New York. 1955-1957.
6. Dylanyan Z. Kh., Arutynuyan Rd.: Biol. Zh. Arm., 1969, **22**, 29.
7. Elliker P. R., Anderson A. V., Hannesson C.: J. Dairy Sci., 1956, **39**, 1611.
8. Homolka L.: Diagnostyka Biochemiczna PZWL, 1961, Warszawa 297.
9. Hempenius W. L., Piska B. J.: J. Dairy Sci., 1968, **51**, 221.
10. Kuila R. K., Ranganathan B., Dutta S. M. Laxminavayana H.: J. Dairy Sci., 1971, **54**, 331.
11. Maksimowa A. K.: Trudy WNIMI., 1970, **27**, 32.
12. Naylor J., Sharpe M. E.: J. Dairy Res., 1958, **25**, 92.
13. Majbaum-Katzenellenbogen W.: Acta Bioch. Polon., 1955, **2**, 279.
14. Ohmiya K., Sato Y.: Agr. Biol. Chem., 1967, **31**, 1318.
15. Ohmiya K., Sato Y.: Agr. Biol. Chem., 1968, **32**, 291.
16. Ohmiya K., Sato Y.: Agr. Biol. Chem., 1969, **33**, 662.
17. Piątkiewicz A.: Acta Alim. Pol., 1976, **2**, (1-2), 73.
18. Piątkiewicz A.: Acta Alim. Pol., 1976, **2**, (3-4).
19. Sharpe M. E.: Dairy Sci. Abstr., 1962, **24**, 165.
20. Szidłowskaja W. N., Diaczenko P. E.: Trudy WNIMI, 1968, 27.
21. Singh Jasjit., Ranganathan B.: Acta Microbiol. Pol., 1974, B.6.1:15.
22. Stadthouders J.: Neth Milk and Dairy J., 1960, **14**, 83.
23. Tourneur C.: J. Dairy Cong., 1970, A.2.3.138.
24. Van der Zant V. C., Nelson F. E.: J. Dairy Sci., 1953, **36**, 1212.
25. Załaszko M. W., Moczałowa K. W.: Moł. Prom., 1969, **3**, 18.

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Authors address: Gdańska 166, 90-537 Łódź.

A. Piątkiewicz, H. Oberman

WPŁYW PROMIENIOWANIA UV ORAZ NITROZOGUANIDYNY NA AKTYWNOŚĆ PROTEOLITYCZNĄ *LACTOBACILLUS CASEI*

Instytut Technologii Fermentacji i Mikrobiologii, Politechnika, Łódź

Streszczenie

Wpływ czynników mutagennych — promieni UV i nitrozoguanidyny na aktywność proteolityczną komórek *L. casei* oceniano na ok. 1000 osobników pochodzących z populacji poddanej działaniu tych czynników. Cechowały się one podwyższoną aktywnością porteolityczną, tj. zdolnością gromadzenia produktów rozkładu białek mleka w porównaniu ze szczepem macierzystym. Stwierdzono dużą heterogenność uzdolnień w tym zakresie, zarówno w populacji macierzystej, jak i poddanej działaniu czynników mutagennych. Zwiększenie homogenności tej cechy uzyskano po kilkakrotnym działaniu czynnika mutagennego. Efekt działania mutagenów wzrastał wraz z dawką promieniowania i stężeniem nitrozoguanidyny. Najlepsze efekty uzyskano przy skojarzonym działaniu obu tych czynników mutagennych, a szczególnie dwukrotnej mutacji skojarzonej mutanta, poddanego powtórnie działaniu mutagenów po 2 miesiącach hodowli laboratoryjnej.

Zastosowane czynniki mutagenne zwiększały również homogenność populacji *L. casei* w zakresie aktywności kwaszącej, eliminując osobniki słabokwaszące, jednakże u wyizolowanych szczepów nie stwierdzono korelacji między aktywnością kwaszącą i proteolityczną. Zmienności różnic między wynikami średnich poszczególnych serii badań mutantów i prób kontrolnych, obliczona w tekście Studenta, wykazała istotne różnice w aktywności proteolitycznej mutantów otrzymanych po działaniu zastosowanych czynników mutagennych w stosunku do szczepu wyjściowego. Pod wpływem czynników mutagennych obserwowano efekt letalny komórek zależny od stężenia i czasu działania mutagenu. Komórki potomne nie wykazywały zmian morfologicznych w stosunku do populacji macierzystej.

Wyizolowane mutanty wykazywały zbliżoną dynamikę wrostu, wyższą aktywność kwaszącą ogólną i lotną oraz duże zróżnicowanie w specyficznej aktywności proteolitycznej surowych ekstraktów enzymatycznych wobec różnych frakcji kazeiny. Większość wyizolowanych szczepów silniej niż szczep wyjściowy trawiła frakcję beta i kazeinę izoelektryczną. Szczep 5/3 wykazywał wyższą aktywność wobec frakcji $alfa_s$, natomiast UV-mutant 60/8, wobec frakcji kapa, przy czym w środowisku rośnie jednocześnie ilość azotu aminokwasowego, co wskazuje na dużą aktywność uwalniania aminokwasów z tego substratu.