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THE EFFECT OF UV-RAYS ON THE PROTEOLYTIC ACTIVITY OF *STREPTOCOCCUS DIACETILACTIS*

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Key words: milk fermentation bacteria, *Streptococci*, mutagenic agents, irradiation, UV-rays, proteolytic activity.

A suspension of *Str. diacetilactis* 239 cells was exposed to UV-rays under standard conditions. The surviving cells showed increased proteolytic activity by 10% to 50% as compared with the parent strain.

The application of active strains of milk fermentation bacteria as starters in dairy industry is well known. Within recent years there has been an increased interest in the proteolytic activity of the bacteria due to a possibility of acceleration by the using proper starter of the process of cheese maturation [9]. Besides, strains with poor proteolytic activity are responsible for a bitter flavour of cheese resulting from accumulation of bitter-flavoured peptides.

The problem of availability of active industrial strains directed many researchers to the task of improving the properties of the strains with active mutagenic agents such as ionizing radiation [11] or UV [8]. These studies indicated the possibility of increase the activity [15] or losing a given physiological feature [8].

The present paper deals with the attempts made towards obtaining mutants of lactic acid streptococci with higher proteolytic activity by applying UV-rays as a mutagenic agent.

MATERIALS AND METHODS

The biological material consisted of strains of *Streptococcus diacetilactis* 239 supplied by the Laboratory of Pure Cultures for Dairy Products in Olsztyn, Poland. The strains were stored at +4°C on skin milk supplemented with chalk and on an acetate medium [1]. Periodically they were

transfered on mm a liquid medium and milk (every 7 to 10 days). The strains were activated by four passages every 24 hours in nutrient media. Incubation temperature — 30°C.

METHODS

1. CULTIVATION OF THE BACTERIA

The strains were cultured in the liquid APLC medium containing dry yeast autolysate — 0.5%; peptone — 1.%; sodium citrate — 1%; lactose — 1.5%. The medium pH — 6.5. The bacteria were cultivated for 7 to 24 hours at 30°C. Determinations were performed on cells from the log growth phase (after seven hours of cultivation) as well as the cells from either the early or the later phases (after 12 or 24 hours of growth). The biological material used in a part of the experiments consisted of the synchronized population of *Str. diacetilactis* 239. The synchronization of growth was obtained by transfer of starved cells onto fresh medium [10]. Under these conditions two to three synchronic divisions were obtained and here the cells used in the experiment derived from the phase prior to division.

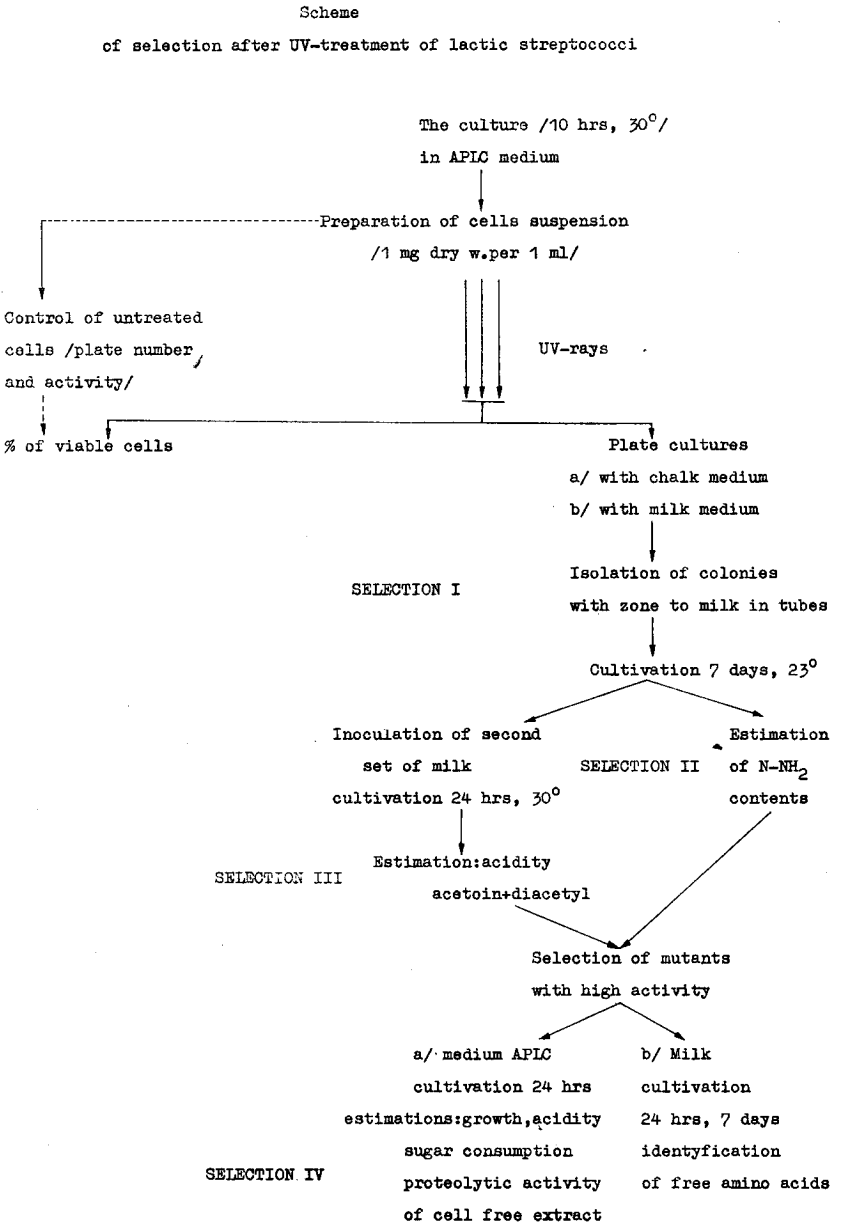
To estimate the survival ability and isolation of the colonies the above mentioned medium supplemented with 2% $CaCO_3$ and 1.5% agar was used. Another medium was composed of agar and milk (20% skim-milk). The milk was reconstituted from powder milk (10% s.m.)

2. MUTATION CONDITIONS UNDER THE UV-RAYS

The cultures were neutralized with sterilized sodium hydroxide solution to pH 6.5. The cells were centrifuged (5000 rpm), washed twice with a sterilized 0.2 M phosphate buffer pH 6.5 and then suspended in the buffer by standardizing the cell density to 1 mg s.m/1 ml, which corresponded to the number of cells from 7.17×18^8 to 1.05×10^9. Radiation was performed on the cells for 15 to 150 seconds in constant standard conditions: the source of rays was a UV bacteriological lamp with a Philips bulb (wave length — 260 nm), with an output of 5.94×10^3 J (sec) 10 ml liquid, the distance between the suspension and the source of rays — 70 cm, liquid volume — 10 ml, liquid layer thickness — ca. 3 mm. During radiation permanent electro-magnetic mixing was applied. The samples were subjected to radiation after a preliminary period of 5 minutes of lamp activation. Following the radiation the suspension was protected against photoreactivation (30° for 90 minutes) and then put on solid media to determine survival ability and isolation of particular colonies. For comparison, a suspension portion not subjected to the irradiation was also cultivated. All of the growths were repeated at least ten times, incubation was continued for 48 hours at 30°.

3. SELECTION OF ACTIVE SPECIES AFTER IRRADIATION

The particular stages of the process of selection of the active strains after exposure to the irradiation are presented in Diagram. The preliminary stages of selection of active species were done on the solid media (the milk medium and the APLC with chalk). The colonies producing a distinct clarification zone (milk proteolysis) were isolated from the



agar/milk medium. The medium with chalk isolated colonies with a distinct sheat, a proff of strong acidifying properties. Species which did not produce clarification on the media were also isolated for comparative purposes. Particular colonies were put on sterile milk and cultivated for 24′ hours at 30°, and then for 7 days 23°. The cultures formed the starting point to the second stage of selection based on assessment of the isolated strains' abilities to digest milk casein. The digestion of casein was determined and assessed in terms of the growth of content of aminoacid nitrogen ($N—NH_2$) in the culture environment in relation to control samples. At the same time comparison was made between abilities of the isolated 24-hour milk culture strains to produce acetoin and to acidify milk. The obtained results were used as the basis of the third stage of selection of the active strains. The active strains from the Irst and IIIrd selection stages were isolated and characterized in greater detail. Their growth as well as their specific features of proteolytic activity against casein and its fractions were assessed, taking into consideration the changes of the aminoacid composition in cultivation during the IVth selection stage [14]. The species with higher proteolytic activity in relation to parent strains were transferred on milk and kept for further research.

4. CONTROL DETERMINATIONS

Survival of the bacteria was determined with the plate method on the basis ot the number of colonies grown on the APLC agar (a) medium or the agan-milk (b) medium after 48 hours of growth at 30°. At least 10 plates wers grown parallelly with every irradiation time and parent suspension. The level of amino groups — after seven days of growth in milk — was determined with the ninhydrine method [5], following precipitation of milk proteins with 12% TCA (trichloroacetic acid). The determination were provided by a spectrocolorimeter Spekol at the wavelength of 570 nm, with the standard curve calibrated for a solution of glycine and glutamic acid. The results are given in mg% of amino-acid nitrogen.

Acidification was determined by titration of 0.1 N NaOH. The results are given in grams of lactic acid per 100 ml culture.

Acetoin and diacetyl were determined with Brandl method [5]. The determination were made on the Spekol spectrocolorimeter, wavelength — 545 nm. The results are given in mg/100 ml culture.

RESULTS AND DISCUSSION

Some 300 clones of non-irradiated populations and 600 clones of irradiated *Str. diacetilactis* were analysed. In the suspension of the UV irradiated bacteria decrease of viability of cells was observed; under fixed experi-

mental conditions the drop in live cells was proportionate to the irradiation time (Fig. 1). When biomass density was approximately the same (ca. 1 mg/ml) the resistance of cells against the UV-raya dropped off with age: the most resistant were the log growth phase cells after 7 hours of cultivation. The lethal effect of radiation grew in the suspension with lower

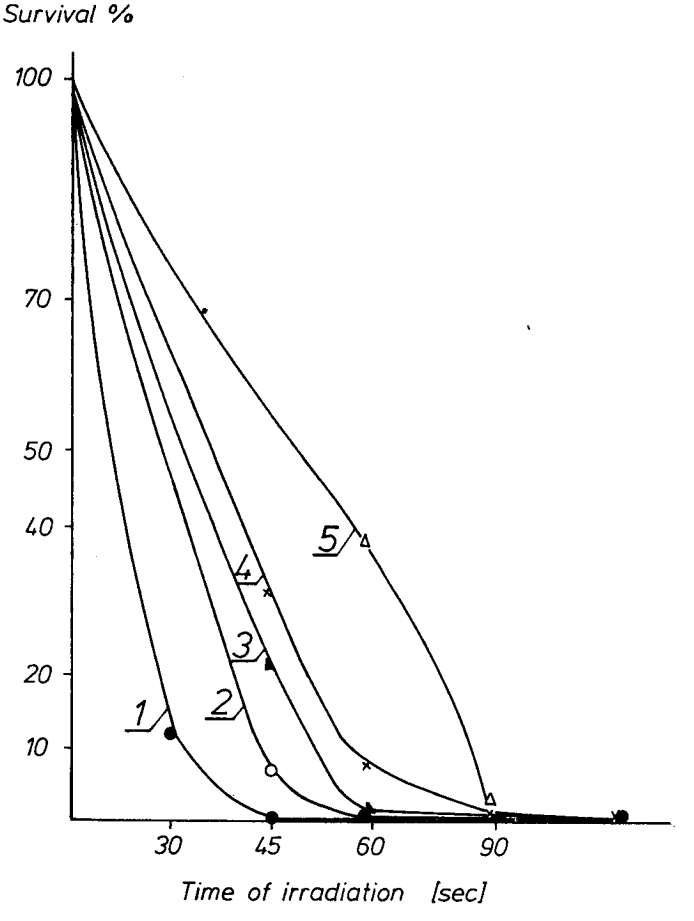


Fig. 1. Survival of *Streptococcus diacetilactis* 239 after UV-treatment; 1 — 13 hrs cultivation, 0,87 mg dry w. per 1 ml; 2 — 24 hrs cultivation, 1 mg dry w. per 1 ml; 3 — 12 hrs cultivation, 1 mg dry w. per 1 ml; 4 — 7 hrs cultivation, 1 mg dry w. per 1 ml; 5 — synchronized cell suspension, 1 mg dry w. per 1 ml

density (0.87 mg s.m./ml). The highest resistance to the UV rays effects was observed in the synchronized cultures. Progeny cells of the irradiated populations did not show any morphological changes in relation to parent population.

Isolated parent strains of *Str. diacetilactis* showed high heterogeneity of the proteolytic activity expressed in an increase of groups N—NH_2

after cultivation in milk. The level of N—NH_2 for *Str. diacetilactis* ranged from 3.7 to 7.3 mg% on the APLC medium and from 2.42 to 6.84 mg% on the milk medium. The obtained results were classified according to the nitrogen (N—NH_2) content: Class I — 2 - 4%; Class II — 4 - 6 mg%, Class III — 6 - 8 mg%, Class IV — 8 - 10 mg% and Class V — 10 - 12 mg%. It was observed that between 90 and 95% of the parent strain porly released aminoacids from milk proteins: less than 2 - 6 mg% (Fig 2). The number of species giving a higher level of nitrogen, about 6 - 8 mg%, were no more than 5 to 10%. The strains releasing more than 6 mg% amino-acid nitrogen in milk were regarded as proteolytically active.

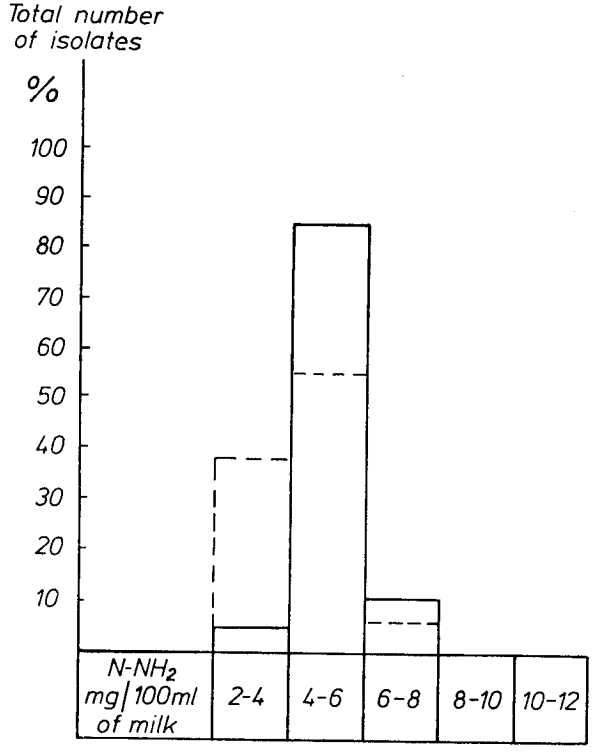


Fig. 2. Proteolytic activity of the parent strain *Streptococcus diacetilactis* 239

The effects of the radiation were tested on some 600 species isolated from the irradiated populations. The irradiated and the parent strains were isolated parallely. Cumulative results of the selection with use of the UV rays in several series are presented in Fig. 3. It can be observed that the populations derived from the irradiated species showed higher potential for caseolysis and release of aminoacid nitrogen in cultures than those of the parent strain.

The number of more active species grew. They released ca. 10 mg% nitrogen, particularly in the population subjected to irradiation with the

UV rays for 60 seconds. Higher doses of irradiation resulted in elimination of weaker substrains, producing no more than 2 - 4 mg% N—NH_2. These tendencies and effects of the UV rays were observed to take place regardless of the growth phase of the biomas used in the experiments.

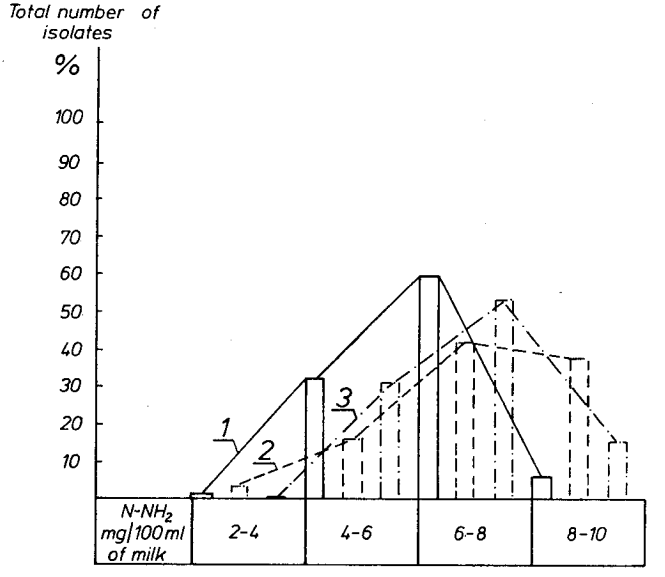


Fig. 3. The effect of UV-irradiation (one treatment) on the proteolytic activity *Streptococcus diacetilactis* 239 (isolates were separated from APLC+$CaCO_3$ medium); 1 — parent strain, 2 — irradiated 60 sec., 3 — irradiated 90 sec.

Analysis of the suspension of *Str. diacetilactis* 239 bacteria enabled observation of a similar effect of the rays, as expressed in a higher number of species providing medium and higher values of N—NH_2, although the tests did not reveal cultures of unusually high proteolytic activity.

Particularly high homogeneity and equal proteolytic activity was displayed by the substrains derived from the population twice time irradiated. Both exposure times, 60 and 90 seconds, eliminated weaker species, which produced results in Classes I and II, that is, at the level of the parent strains. Also a distinct increase in the number of species cumulating higher quantities of amino acid nitrogen after cultivation in milk were observed. In the milk-agar media the irradiation produced a increased number of species that effected the clearing zones around colonies, particularly at higher exposure times; from 50% in the parent strain population to about 70% species after irradiation within 90 seconds (Table 1). Longer exposure times also accounted for a higher percentage of the species with acidifying properties assessed in the medium with chalk — from 37% for the parent population to 47% for the population irradiated within 90 seconds. Re-

Table 1. Protcolytic activity of mutants *Streptococcus diacetilactis* 239 after UV-treatment
A. Expressed as a zone on milk agar
B. Exprcssed as N—NH_2 content in milk

Time of irradiation sec	Total number of isolates	A. Colonies with a zone total number of colonies %	Range	B. N—NH_2 mg/100 ml of milk					
				from 2 to 4 %	from 4 to 6 %	from 6 to 8 %	from 8 to 10 %	from 10 to 12 %	from 12 %
parent strain	18	50	2.42 - 6.84	39.2	55.5	5.3	—	—	—
60	25	56	4.48 - 12.54	0	16	48	16	8	12
90	29	69	4.9 - 10.72	0	17.3	58.7	20.5	3.5	—

peated irradiation revealed species with high proteolytic potential producing results within Class IV and Class V, that is about 10 - 14 mg% aminoacid nitrogen. The irradiated population showed also evened out features of milk acid-ification. All samples after 24 hours of cultivation produced homogenous coagulation of milk. It was observed, however, that in the case of *Str. diacetilactis* strain the level of aminoacid nitrogen did not correlate in absolutely close ways with acidifying properties or the size of the casein proteolysis zone in the agar-milk medium (Table 2). The species providing very poor clearing zones around the colonies in the milk medium — a proof of poor caseolysis — cumulated large quantities of aminoacid nitrogen during cultivation in milk, and reversely. The irradiation time was of no special significance here.

No absolute correlation was observed also between the acidifying, proteolytic and aroma-producing properties of the bacteria before and after irradiation (Table 3). Assessing these properties in more than 100 samples (tests) it was determined that the species with a high proteolytic potential were characterized by poorer productivity of acetoine and low ability to acidify milk. It was also observed that the best acidifying strains were never among the poorest proteolytically.

Proteolytic activity of the analysed strains of milk streptococci, that is, the ability for digesting casein plus release of free aminoacids, turned out to be a very heterogenic feature. In the population there were species with very poor potential for accumulating aminoacids in cultivation and there were also more active speciec. Fluctuations in the level of aminoacid nitrogen in cultivation, depending on the strains, were observed by many researchers of the problem [7, 9, 12]. It was noticed that higher activity was a characteristic feature of lactobacilli. In this studies were lack, however, of analysis of population abilities.

In effect of the influence UV-rays on the suspension of *Str. diacetilactis* higher potential for relaease of aminoacids in milk cultivation was observed. The irradiation eliminated from the population the species that were poor and had low proteolytic activity. The method may be, therefore, considered a way for induction of proteolytic activity in there strains. Similar effect of the UV-rays were observed in numerous studies conducted recently to select active milk bacteria strains for dairy industries [2, 3, 13].

The analysis did not prove any close relationship between the acidifying, proteolytic and arome-producing abilities of the strain. The data concerning this problem in the literature are controversial. For some strains a close dependence between the acidifying and proteolytic properties was observed; strongly acidifying strains caused also high proteolysis of casein. It was determined for *Str. lactis* and *Str. diacetilactis* [16] but it was not observed in *Str. cremoris* and in milk bacilli. It seems, however, that the selection of the proteolytically active species for industrial

Table 2. The effect of UV-irradiation on the acidifying and proteolytic activity of *Streptococcus diacetilactis* 239

Parent strain			Time of UV-tretament					
			60 sec			90 sec		
Colonies with zone on milk agar	Curdling of milk	mg N—NH_2 100 ml of milk	Colonies with zone on milk agar	Curdling of milk	mg N—NH_2 100 ml of milk	Colonies with zone on milk agar	Curdling of milk	mg N—NH_2 100 ml of milk
+	+	3.45	+	+	7.2	++	+	4.9
+	+	4.06	+	+	4.48	++	+	5.02
+	+	3.7	+	+	7.38	++	+−	6.06
+	+−	4.48	+	+	7.56	+	+	5.48
+	+	6.84	+	+	6.81	+	+	9.32
−	+	5.46	+	+	9.62	+	+	10.72
−	+	3.82	−	+	9.14	++	+	7.32
−	+	4.6	−	+	12.42	−	+	9.0
−	+	2.42	−	+	10.3	−	+	9.62
−	+	3.54	−	+	5.94	−	+	6.48
−	+	4.66	−	+	12.54	−	+	6.0

Table 3. The effect of UV-irradiation on the proteolytic activity, acidifying and aroma-forming *Streptococcus diacetilactis* 239

Parent strain			Time of UV-treatment					
			60 sec.			90 sec.		
Lactic acid g/100 ml of	Diacetyl and acetoin mg/100 ml of milk	mg N—NH_2 100 ml of milk	Lactic acid g/100 ml of milk	Diacetyl and acetoin mg/100 ml of milk	mg N—NH_2 100 ml of milk	Lactic acid g/100 ml of milk	Diacetyl and acetoin mg/100 ml of milk	mg N—NH_2 100 ml of milk
0.86	10	4.4	0.87	9.5	6.2	0.88	10	6.6
0.78	10	4.0	0.88	10	6.8	0.92	11	7.6
0.87	11	4.4	0.86	9.8	8.0	0.87	9	7.6
0.88	9.5	3.2	0.89	12.5	4.8	0.87	10	7.2
0.79	10	3.0	0.84	11	4.8	0.92	10.5	9.0
0.83	10	3.2	0.88	10	4.8	0.81	10.5	5.6
0.86	11	3.2	0.82	—	5.4	0.82	12.5	5.8
0.82	8.5	3.0	0.90	10	5.2	0.88	10	5.6
0.84	8.7	3.2	0.82	11.5	2.0	0.82	13	7.4
0.84	8.7	3.2	0.90	10	4.0	0.85	12.0	6.0

purposes must be related to selection of properly acidifying strains. Although in the experiments under discusion no absolute correlation was observed in this regard but in most cases the properly acidifying strains revealed good or medium proteolytic activity. Thus, the linking of these features and potential in terms of industrial needs is fully justified and it may provide strains which, next to carry out a correct technological process will also accelerate the process of protein degradation and cheese ripening.

CONCLUSIONS

1. Proteolytic activity of the parent population *Str. diacetilactis* is a highly heterogenic feature.
2. Susceptibility of streptococci cells to mutagenic agents is variable and depends on the time of exposure to UV radiation, as expressed in the differentiated degree of cell survival.
3. The UV-rays produced an increase of proteolytic activity of strains surviving to 10 to 50%.
4. The increase in proteolytic activity of the UV mutagenized populations was proportionate to the dose of radiation.
5. The UV rays used in standard conditions eliminated from populations the species with lowest proteolytic activity thus increasing homogenity of the populations in this respect.
6. Mutagenization of *Str. diacetilactis* increased the acidifying property of the surviving species. It was observed that the weak individuals undergo elimination.
7. No correlation between acidifying and proteolytic properties in *Str. diacetilactis* population was observed.

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WPŁYW PROMIENI UV NA AKTYWNOŚĆ PROTEOLITYCZNĄ *STR. DIACETILACTIS*

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Streszczenie

W celu uzyskania aktywnych proteolitycznie szczepów paciorkowców mlekowych zastosowano promieniowanie ultrafioletowe. Działaniu promieni UV poddawano standaryzowaną zawiesinę bakterii o gęstości ok. 1 mg s.m. w 1 ml, pochodzących z różnych faz wzrostu. Selekcję aktywnych proteolitycznie szczepów prowadzono w hodowli w mleku, oznaczając przyrost azotu aminowego. Porównawczo oznaczano aktywność kwasotwórczą i aromatyzującą tych szczepów. Przebadano ok. 300 osobników populacji wyjściowej i ok. 700 populacji napromieniowanych. Stwierdzono dużą heterogenność aktywności proteolitycznej populacji macierzystej. Faza wzrostu komórek poddawanych napromienianiu nie wpływała na efekt ich działania. Wpływ promieni UV wyrażał się wyeliminowaniem szczepów o najniższej aktywności proteolitycznej oraz zwiększeniem liczby szczepów aktywnych o 10 - 50%. Szczepy o najwyższej aktywności proteolitycznej izolowano po dłuższym czasie naświetlania, przy przeżywalności rzędu 0,05%. Nie stwierdzono korelacji uzdolnień kwaszących, aromatyzujących i proteolitycznych u badanych szczepów.