STUDY OF THE INFLUENCE OF *SAVINASE®EVITY 16L* ENZYME ON BIOFILMS FORMATION OF *STAPHYLOCOCCUS AUREUS* ON STAINLESS STEEL WITH DIFFERENT ROUGHNESS

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Abstract

Microbial films formation on the dairy equipment creates a serious problem, because they are difficult to eliminate by washing and disinfecting means that results in contaminating dairy products by microorganisms. The aim of the work was to study the influence of Savinase®Evity 16L proteolytic enzyme on the process of destructing biofilms, formed by Staphylococcus aureus on stainless steel with different surface roughness.

It has been established, that surface roughness of stainless steel influences the process of Savinase®Evity 16L enzyme penetration in a hollow and prevents the destruction of the biofilm matrix, created by Staphylococcus aureus.

It has been revealed, that after the influence of a proteolytic enzyme on Staphylococcus aureus biofilms, created on steel with roughness 0,16±0,018 mcm, the density decreased in 4,0 times ($p \leq 0,05$), comparing with a condition before processing. At roughness 0,63±0,087 mcm the density of formed biofilms decreased at the effect of Savinase®Evity 16L in 3,3times ($p \leq 0,05$) and the biofilm was characterized as a weak one. At the same time at stainless steel surfaces with roughness 2,68–0,95mcm, the

density of biofilms decreased in 2,3–2,1 times ($p \leq 0,05$), comparing with a condition before processing, and they were characterized as ones of the middle density. It has been also revealed, that the degradation intensity of biofilms under the influence of Savinase®Evity 16L enzyme at roughness 2,68–0,95 mcm was 1,7–1,9 times ($p \leq 0,05$) lower than at the surface with roughness 0,16±0,018 mcm.

So, the revealed degradation features of a biofilm, created by Staphylococcus aureus at surfaces of stainless steel of different roughness at the influence of Savinase®Evity 16L proteolytic enzyme give a possibility to substantiate the addition of proteolytic enzymes to the composition of washing means for dairy production. It is also offered to process the surface to the roughness no more than 0,63 mcm for producing food steel for raising the effectiveness of biofilms destruction by enzymes and for the sanitary processing.

Keywords: enzyme Savinase®Evity 16L, destruction, density of biofilms, roughness of dairy equipment, sanitary processing.

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1. Introduction

The main operation in the production technology of safe dairy products is the introduction of effective sanitary processing regimes (washing and disinfection) for the whole dairy equipment [1]. The sanitary processing of the technological equipment is realized at such level, at which all, remained on the internal surface (dairy-fatty residues, microorganisms), don't create a threat for safety of ready products [2]. But despite the introduction of the whole complex of sanitary-hygienic requirements as to eliminating microorganisms at the technological equipment and in ready products, such as washing, disinfection, thermal processing, an effective result is not always achieved [3]. It is connected with a fact that microorganisms survive on the technological equipment due to the ability to form biofilms and also stable strains of microorganisms remain at the continuous sanitary processing [2, 4, 5]. At the same time literary data indicate [6] that wear and tear of the stainless steel surface in dairy production change roughness that worsens the washing process, in such a way raising microbial adhesion. Organic substances accumulate in worn out surfaces of stainless steel; they increase a surface area for contacts between microorganisms and also are a food nutritive medium for the development [7, 8]. It is accepted, that such elements of the surface topography as scratches, hollows, protrusions and cracks play an important role in the process of adhesion and formation of a biofilm by microorganisms [9, 10]. According to data [11], the equipment, where only one plankton bacterium is found, contains near 1000 microorganisms, formed in biofilms. That in why for today scientists pay the essential attention to studying biofilms on the dairy equipment, because the biofilm matrix composition may include pathogenic bacteria, resistant to washing and disinfecting means [12–14]. The studies demonstrate that after fixing to the abiogenic surface, bacteria become to multiply, creating a multicellular layer (cellular clusters), included in the polymer matrix that forms a microbial biofilm [15, 16]. The formed biofilm matrix mainly has the glycopolypeptide construction [17, 18], for eliminating which from the dairy equipment, the standard sanitary processing by alkaline and acid washing means is not always effective [19]. So, it is urgent to conduct studies for investigating a possibility of destructing biofilms, formed on the dairy equipment using enzymes. According to data [20] the topicality of the studies is in fact that enzymes must destruct biofilms, formed on the equipment, and the following operation of the sanitary processing – disinfection – must eliminate microbial cells. But the use of enzymes must be accompanied by detail studies of the influence on microorganisms that contaminate an object and chemical composition of the biofilm matrix. For today the most group of industrial substances for washing means in food industry is of the protease class [21]. It is connected with a fact that contaminations on the technological equipment consist mainly of fat and protein, at the same time proteins are included in the matrix of microbial biofilms. So, the studies about the effectiveness of proteolytic enzymes as to the influence on formed biofilms of microorganisms, separated in dairy industry are topical and promising. The aim of the work was to study the influence of Savinase®Evity 16L proteolytic enzyme on the process of destructing biofilms, formed by Staphylococcus aureus on stainless steel with different surface roughness. It allows to ground scientifically the activity of proteolytic enzymes in the composition of washing means, depending on possible surface roughness.

2. Materials and Methods

2. 1. Studied materials and equipment, used in the experiment

Plates of stainless corrosion-stable steel of the trademark AISI 321, with size 30×30 mm and width 5 mm, surface roughness 2,687±0,014 mcm, 0,95±0,092 mcm, 0,63±0,087 mcm and 0,16±0,018 mcm **(Fig. 1)** were used in the work. The surface roughness of the plates of stainless steel was determined using the profilometer of 296 mark. The process of biofilm formation was studied on a strain model of *Staphylococcus aureus ATCC 25923 (S. aureus).*

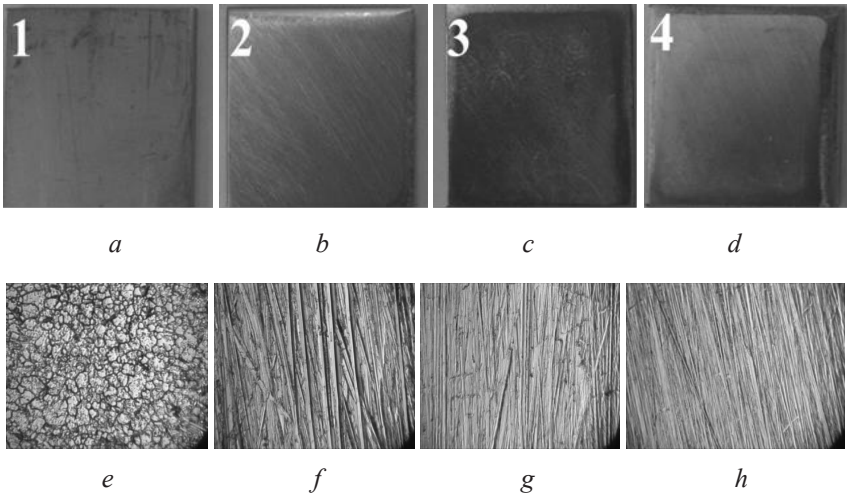


Fig. 1. Outlook of the plates of stainless steel AISI 321 with different roughness:
a, b, c, d – native outlook of the plates; *e, f, g, h* – outlook of the plates under the microscope (magnification 1500 times)

The microscopic studies of biofilms on stainless steel were realized on the electric raster microscope (REM 106 I, Ukraine) (**Fig. 2**).

Fig. 2. Electric raster microscope (REM 106 I)

The modern electric microscope with the system of energy-dispersive microanalyzer allows to get high-quality electric-optic images in the regime of secondary electrons.

4. 2. Methods of determining microbiological parameters of the equipment microflora

The density of the formed microbial biofilms was determined in the following way. Sterile plates of stainless steel with the correspondent surface roughness were set in sterile Petri dishes, then there was added sterile meat-peptone broth (MPB) and test-culture *S. aureus* with a concentration as in average 1010 thousand of cells for 1 cm^2 of the plate area. In 24 hours of incubation

at temperature 25 °C the plates were taken out from the Petri dishes, washed off from plankton (unfixed) microorganisms by the phosphate buffer threefold. Then the plates were covered by *Savinase®Evity 16L* proteolytic enzyme (Novozimes) in 0,05 % concentration at temperature 60±2 °C and kept during 30 min. After that the enzyme solution was washed off by the phosphate buffer, and the formed biofilms were fixed by 96° ethyl alcohol. After fixing, the biofilms were colored in 0,1 % crystal violet solution and dried. Then each plate was separately poured by 7,0 cm^3 of 96° ethyl alcohol and left for 10 min. After exposition for 10 min, 5 cm^3 of the washing solution were taken from biofilms, and the optic density was determined spectrophotometrically at wave length 570 nM on the photometer CPC-3 (Ukraine) **(Fig. 3)**.

Fig. 3. Photometer (CPC-3)

At the optic density of the washing solution up to 0,5 un., the density of the formed biofilms was considered as low and it was accepted, that steel demonstrates perfect anti-adhesion properties; at 0,51–1,0 un. – middle, good anti-adhesion properties of the steel surface; at solution density 1,01–1,30 un., the density of the formed biofilms was considered as high, anti-adhesion properties of steel are satisfactory; more than 1,31 – very dense, unsatisfactory anti-adhesion properties of steel [2].

3. Results

The formed and colored biofilms of *S. aureus* on the plates with different surface roughness, in the control before processing and after processing by 0,05 % solution of *Savinase®Evity 16L* enzyme at temperature 60±2 °C during 30 min are presented on **Fig. 4.**

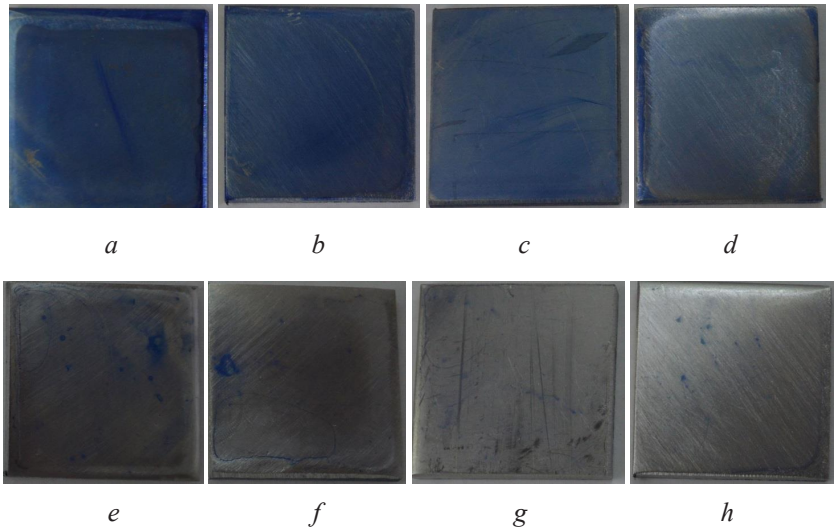
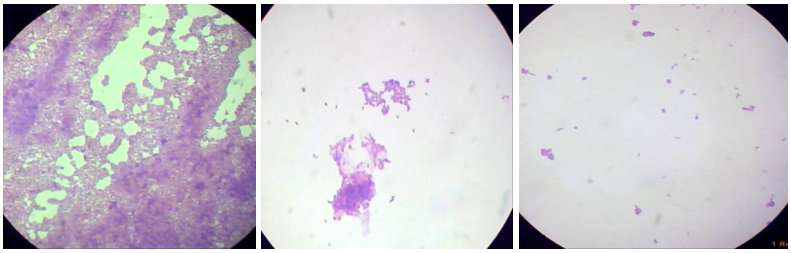


Fig. 4. Outlook of the stainless steel plates of different roughness, on which the biofilms of *S. Aureus* are formed *a, b, c, d* – before processing by *Savinase®Evity 16L* enzyme; *e, f, g, h* – after processing by the enzyme

The data on **Fig. 4** demonstrate that after processing by the *Savinase®Evity 16L* enzyme there takes place the biofilm matrix degradation. But the plates of surface roughness 0,63–0,16 mcm (**Fig. 4, *g, h*)** were cleaner, comparing with the ones with roughness 2,68–0,95 mcm (**Fig. 4, *e, f***). It indicates that the surface roughness influences the process of enzyme penetration in roughness hollows and prevents the biofilm matrix destruction.

Fig. 5 presents the microscopic image of the biofilm conditions before processing (**Fig. 5, *a***) and biofilms degradation under the influence of *Savinase®Evity 16L* enzyme, (**Fig. 5, *b*** – on the steel plates with roughness 0,63±0,87 mcm and **Fig. 5, *c*** – at roughness 0,16±0,018 mcm).



a *b* *c*

Fig. 5. Microscopic image of the biofilm conditions on the plates with different surface roughness: *a* – before processing by *Savinase®Evity 16L* enzyme, *b* – after processing by the enzyme with surface roughness 0,63±0,87 mcm; *c* – at roughness 0,16±0,018 mcm (magnification 1350 times)

As it can be seen on **Fig. 5, *a*,** the biofilm, formed of *S. aureus* looks as an integral conglomerate that fills the whole plate surface. After the influence of *Savinase®Evity 16L* proteolytic enzyme the matrix destruction took place (**Fig. 5, *b, c***), in which result the number of microbial cells in the plate surface biofilm essentially decreased.

The results of the studies of the influence of *Savinase®Evity 16L* proteolytic enzyme at 0,05 % concentration during 30 min on the biofilms, formed of *S. aureus* by the stainless steel surfaces of different roughness are presented in **Table 1.**

Table 1

Influence of *Savinase®Evity 16L* enzyme on the biofilms of *S. aureus*, formed on stainless steel with different surface roughness

Roughness of stainless steel plates, mcm	Density of biofilms, formed of *S. aureus*, un.		Characteristics of biofilms after processing
	Before processing by enzyme	After processing by *Savinase®Evity 16L* enzyme	
0,16±0,018	1,37±0,09	0,34±0,03*	weak
0,63±0,087	1,49±0,09	0,45±0,04*	weak
0,95±0,092	1,68±0,11	0,71±0,05*	middle
2,687±0,014	1,92±0,13	0,90±0,05*	middle

*Note: * – p≤0,05 – comparing with the biofilms density before processing by the enzyme*

As it is seen from the data, presented in **Table 1**, the effectiveness of *Savinase®Evity 16L* enzyme on the biofilms, formed of *S. Aureus* decreased on the stainless steel surfaces with the high density. After the influence of the proteolytic enzyme on the biofilms of *S. aureus,* formed on steel with roughness 0,16±0,018 mcm, the density decreased in 4,0 times (p≤0,05), comparing with the condition before processing. The density of the formed biofilms on the stainless steel plates with roughness 0,63±0,087 mcm decreased under the effect of *Savinase®Evity 16L in* 3,3 times *(*p≤0,05) and the biofilm was characterized as weak. The least degradation of biofilms under the influence of the enzyme was revealed on the plates with roughness 2,68–0,95 mcm. On

these steel surfaces the biofilms density decreased in 2,1–2,3 times (p≤0,05), comparing with the condition before processing, and they were characterized as ones of middle density. At the same time the degradation intensity of the biofilms at roughness 2,68–0,95 mcm was in 1,7–1,9 times (p≤0,05) less than on the surface with roughness 0,16±0,018 mcm. It indicates that the prophylactic treatment by means with proteolytic substances must be conducted for fighting against microbial biofilms on the dairy equipment and for increasing the effect of the sanitary processing. The studies have also revealed that the roughness increase of the stainless steel surface favors the effectiveness decrease of *Savinase®Evity 16L* proteolytic enzyme. It is probably connected with a fact that microbial cells are better protected from the catalytic effect of the enzyme in roughness hollows. At that, the obtained data are the scientific background for studying the influence of other groups of enzymes (lipolytic, glycolytic) on the biofilm matrix, formed by different types of microorganisms. It allows to substantiate the development of complex washing means for dairy industry with adding enzymes to the concrete technological line of the production process. The obtained data also indicate a necessity of using stainless steel with the low roughness (less 0,63 mcm) for decreasing the formation of dense biofilms and for increasing the effectiveness of washing means.

4. Conclusions

It has been established, that surface roughness of stainless steel influences the process of Savinase®Evity 16L enzyme penetration in a hollow and prevents the destruction of the biofilm matrix, created by Staphylococcus aureus.

It has been revealed, that after the influence of a proteolytic enzyme on Staphylococcus aureus biofilms, created on steel with roughness 0,16±0,018 mcm, the density decreased in 4,0 times (p≤0,05), comparing with a condition before processing. At roughness 0,63±0,087 mcm the density of formed biofilms decreased at the effect of Savinase®Evity 16L in 3,3times (p≤0,05) and the biofilm was characterized as a weak one. At the same time at stainless steel surfaces with roughness 2,68–0,95mcm, the density of biofilms decreased in 2,3–2,1times (p≤0,05), comparing with a condition before processing, and they were characterized as ones of the middle density. It has been also revealed, that the degradation intensity of biofilms under the influence of *Savinase®Evity 16L* enzyme at roughness 2,68–0,95 mcm was 1,7–1,9 times (p≤0,05) lower than at the surface with roughness 0,16±0,018 mcm.

So, the revealed degradation features of a biofilm, created by Staphylococcus aureus at surfaces of stainless steel of different roughness at the influence of Savinase®Evity 16L proteolytic enzyme give a possibility to substantiate the addition of proteolytic enzymes to the composition of washing means for dairy production. It is also offered to process the surface to the roughness no more than 0,63 mcm for producing food steel for raising the effectiveness of biofilms destruction by enzymes and for the sanitary processing.

So, the revealed features of degradation of a biofilm, created by Staphylococcus aureus at surfaces of stainless steel of different roughness at the influence of a proteolytic enzyme Savinase®Evity 16L give a possibility to substantiate the addition of proteolytic enzymes to the composition of washing means for dairy production. It is also offered to process the surface to the roughness no more than 0,63 mcm for producing food steel for raising the effectiveness of biofilms destruction by enzymes and for the sanitary processing.

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