BIOFILM AND THE USE OF PROTEOLYTIC ENZYMES AT SANITATION OF MILKING MACHINES

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Introduction

After the deposit of organic and inorganic molecules on the surface (*Hood and Zottola, 1995*; *Wilderer et al., 1989*), micro-organisms attach themselves to the surface containing food. The inseparable attachment to the surface is followed by the formation of micro-colonies, protected from the influences of the environment through the growing of the exopolysaccharide protective layer, forming biofilm (*Characklis and Marshall, 1990*). Due to its persistence and resistance, biofilm is unaffected by the normal cleaning agents. The researches also mention the use of enzymatic agents in the sanitation processes. Enzymes are interesting due to their efficient action at low temperatures and low concentrations, because they are degradable and disintegrate to environment-friendly decomposition substances (*Flint et al., 1999; Pintarič, 2002*).

Material and methods

The test studied the effect of using proteolytic enzymes in the cleaning of milking machines. To this end, we used a milking machine to which test segments – that we were able to take off optionally – were attached in the tubular part of the long milking tube. Test segments were exposed to milk flow during milking. After milking, the test segments were removed from the milking machine and alternatingly attached for individual manners of cleaning.

The process of cleaning the milking machine included the rinsing of milk remains with water, the main cleaning with a cleaning agent and the final rinsing with water. The whole cleaning process took 40 minutes.

Test solutions

In order to determine the effects of sanitation with proteolytic enzymes, for the purpose of comparison, during cleaning test plates were exposed to:

- running water without cleaning agents,
- test solution of proteolytic enzymes (> 5 % of serine peptidase, 15-30 % non-ionised tensides) and surfactants (5-15 % phosphates, 15-30 % carbonates, 1-5 % potassium hydroxide) (**PS**), and
- alkali (25-50 % sodium hypochlorite, 10-25 % potassium hydroxide, 2.5-10 % natron sodium silicate) and acid test solution (25-50 % phosphoric (5) acid, 2.5-10 % sulphurous (6) acid > 2.5 % cations) (**AA**).

In all cases, the sanitation process took place under equal conditions.After the cleaning process was finished, the test segments were removed and smears were taken from them for microbiological analysis and determination of ATP presence. Eluates were taken from the segments by means of sterile physiological solution to be checked for the presence of micro-organisms and the value of ATP in them. After sampling, the test segments were again attached to the milking system, where they were again poured over with milk directly during milking.

The test took 21 days. For each sample, the presence of micro-organisms and ATP value were determined in smears and eluates. Measurements were made in two replicates. A total of 84 samples were taken.

Taking and preparation of smears for microbiological investigation

Smears were taken from the entire surface of test segments by means of sterile swabs, soaked in sterile physiological solution. In the laboratory, the smears taken were first well stirred in the shaker. Then, a sterile pipette was used to take the suspension, which was applied directly to petri dishes or it was first diluted in the physiological solution in the ratios from 10^{-1} do 10^{-4}. The suspensions prepared were applied to petri dishes and poured over with milk agar (Oxoid, England). This was followed by the incubation of the samples for 72 hours at 30°C±1. The data were presented as colony units (CFU).

In the preparation of eluates, the interior of the test segments was eluted after the cleaning process with 10 ml of sterile physiological solution. The eluate was then examined for the presence of micro-organisms using the same procedure as for the smears taken.

Measurement of ATP-bioluminiscence

In order to measure the ATP-bioluminiscence on the test surfaces, we used the smears taken after cleaning according to the instructions of the producer (Charm Sciences Inc., USA). We also measured the amount of ATP in test solutions after cleaning. The apparatus Luminator T® (Charm Sciences Inc., USA) was used for measuring. In addition to the preparation of eluates for microbiological researches, eluates were also examined for ATP value for the same producer and by means of the same measurement device.

Results

Micro-organism counts in smears taken from the segments of the test milking machine

After the sanitation process, samples were taken from the segments of the test milking machine by means of smears to be used for microbiological analysis.

The results of the microbiological analyses showed occasional presence of micro-organisms on test segments, which was why the statistical analysis of data was not made.

The values of micro-organism counts in the eluates from the segments of the test milking machine

After the sanitation process, samples were taken from the segments of the test milking machine by means of eluates to be used for microbiological analysis. The average values of micro-organism counts are shown in the decimal logarithm per cm^2 ($\log_{10}(X)/cm^2$) in table no. 1.

Table 1: *Average values of micro-organism counts (log_{10}) in the eluates from the segments of the test milking machine and their mutual comparisons (n=21)*

		Water (1.42 CFU/cm^2)	**PS** (1.11 CFU/cm^2)
PS (1.11 CFU/cm^2)	diff. diff. % sig.	-0.31 -21.83% NS	*
AA (1.47 CFU/cm^2)	diff. diff. % sig.	0.05 3.52% NS	**0.36** **32.43%** **(P<0.05)**

Note: NS – no statistically significant difference

Measurement of ATP smears taken from the segments of the test milking machine

After the cleaning process, samples were taken from the segments of the test milking machine by means of smears to be used for the determination of the ATP value. The results are presented in table no. 2.

Table 2: *Average ATP values on the segments of the test milking machine and their mutual comparisons (n=21)*

		Water (1,511.00 RLU)	**PS** (1,966.32 RLU)
PS (1,966.32 RLU)	diff. diff. % sig.	455,32 30.13% NS	*
AA (1,988.64 RLU)	diff. diff. % sig.	477,64 31.61% NS	22,32 1.14% NS

Note: NS – no statistically significant difference

Measurement of ATP in the eluates of the segments of the test milking machine

After the cleaning process, ATP was measured in the eluates of the segments. The results of measurements are presented in table no. 3.

Table 3: *Average ATP values in the eluates of the segments of the test milking machine and their mutual comparisons (n=21)*

		Water (19.45 RLU)	**PS** (173.91 RLU)
PS (173.91 RLU)	diff. diff. % sig.	154,46 794.14% NS	*
AA (89.23 RLU)	diff. diff. % sig.	69,78 358.77% NS	-84,68 -48.69% NS

Note: NS – no statistically significant difference

Discussion

The test compared the possibility of using the proteolytic cleaning method with surfactants for the cleaning of a milking machine in cleaning by means of warm water. The following of the presence of micro-organisms by means of taking smears showed occasional increases in the number of micro-organisms on the surfaces of the test segments. Although it was not possible to statistically determine the difference in the count of micro-organisms between individual methods of cleaning, chart 1 shows a count of micro-organisms for PS that is, on the average, 89.74% lower than for the classical AC cleaning method. The best result has also been found in the determination of micro-organisms in the eluates of the test segments. Also in eluates, the values of micro-organism counts determined were the lowest for PS cleaning.

Along with the taking of smears for the determination of the presence of micro-organisms on test segments, smears were also taken in order to determine the ATP value by means of bioluminescence. The results indicate a similar effect of both manners of cleaning (PS=1,966.32 RLU; AC=1,988.64 RLU; P>0.05).

Surprisingly, the results of control segments, only cleaned with water, show lower values than the segments cleaned by means of the PS and AC methods. A possible explanation for the results achieved is in the formation of biofilm on the surface. With biofilm, the resistance of protective exopolysaccharide layer prevents smears to be taken from the surface, which could be the reason for the results obtained. A similar situation was found in the determination of ATP values in eluates, where the lowest average ATP value was also established in control eluates. In order to confirm the hypothesis, in further research it will be necessary to use other methods (for instance, fluorescent coloration and the use of confocal laser microscopy), able to prove the presence of biofilm on the surface. It will also be necessary to study the possibility of removing biofilm on the surface by means of agents like the peroxiacetic acid and its efficiency in removing the exopolysaccharide protective layer.

Conclusions

The comparison of the results of the microbiological representation as well as the presence of ATP in smears and eluates indicate the same or better results in cleaning by means of PS compared to the AC cleaning method. The advantages of the PS method of cleaning are also shown in the lower temperature of the cleaning agent needed, which means that less energy is necessary for the warming up of the cleaning solution. Particularly in the winter period, the PS cleaning is a more reliable way of cleaning a milking machine. An important advantage of cleaning with PS is also the 10-times lower concentration of the cleaning agent (0.04%) and the ready degradability of the active substance. Lower concentrations of cleaning agents and ready degradability reduces the burdening of the environment by the decomposition products of the cleaning agents.

Literature

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