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Resistance of Bacterial Biofilms Formed on Stainless Steel Surface to Disinfecting agent

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Abstract

The natural ability of microorganisms for adhesion and biofilm formation on various surfaces is one of the factors causing the inefficiency of a disinfection agent, despite its proven activity *in vitro*. The aim of the study was to determine the effectiveness of disinfecting substances on bacterial biofilms formed on stainless steel surface. A universally applied disinfecting agent was used in the tests. Bacterial strains: *Listeria innocua*, *Pseudomonas putida*, *Micrococcus luteus*, *Staphylococcus hominis* strains, were isolated from food contact surfaces, after a cleaning and disinfection process. The disinfecting agent was a commercially available acid specimen based on hydrogen peroxide and peroxyacetic acid, the substance that was designed for food industry usage. Model tests were carried out on biofilm formed on stainless steel (type 304, no 4 finish). Biofilms were recorded by electron scanning microscope. The disinfecting agent in usable concentration, 0.5% and during 10 minutes was ineffective for biofilms. The reduction of cells in biofilms was only 1–2 logarithmic cycles. The use of the agent in higher concentration – 1% for 30 minutes caused reduction of cell number by around 5 logarithmic cycles only in the case of one microorganism, *M. luteus*. For other types: *L. innocua*, *P. putida*, *S. hominis*, the requirements placed on disinfecting agents were not fulfilled. The results of experiments proved that bacterial biofilms are resistant to the disinfectant applied in its operational parameters. Disinfecting effectiveness was achieved after twofold increase of the agent's concentration.

Key words: adhesion, biofilm, disinfecting agent

Introduction

Settlement of microorganisms on abiotical surfaces is a very common process in various spheres of life. Solid surfaces that are in contact with water environments are the subject of microbial colonization in the first place. The adhesion of single microorganisms to the solid surfaces gives rise to the formation of a specialized cell culture, called a biofilm.

Because of plentiful nutriments and water, the food processing environment is particularly susceptible to biofilm formation. The presence of *Listeria monocytogenes*, *Bacillus cereus* or *Streptococcus thermophilus* cells on food contact surfaces is about 500 to 50 000 times rarer than the biological films forming by these bacteria (Zottola and Sasahara, 1994). This phenomenon can be the direct cause of product organoleptic changes and food deterioration. Moreover, the adhesion of microorganisms to the solid surfaces may cause food contamination by pathogenic and spoilage microorganisms (Pontefract, 1991).

In the food industry, one of the fundamentals of internal control and good manufacturing and hygienic production (GMP/GHP) are regular cleaning and disinfection procedures, since food safety and quality is determined by the efficacy of disinfecting agents. Literature sources report that biofilm formation by some bacteria can take only 2–4 hours (Yuehuei *et al.*, 1997). Our research confirmed this observation as we noted that *Pseudomonas putida* and *Staphylococcus hominis* formed a biofilm after 4 hours of incubation on a stainless steel surface. For practical and

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economical reasons, in manufacturing plants frequent execution of complex hygienic procedures is impossible. Moreover, the time between particular cleaning and disinfection cycles is from several hours to a few days and that promotes the process of biofilm formation. Thus, there is a need for the adaptation of proper procedures and application of agents that enable the efficient eradication of these bacteria.

The effectiveness of hygienic procedures depends mostly on the right choice of cleaning and disinfecting agents. The so far available antibacterial agents may show lower activity towards phenotypically altered sessile bacteria, since they were developed and introduced into production based on determined high activity against planctonic population of microorganisms using classical measurements – minimal ihibitory concentration (MIC) and minimal bactericidal concentration (MBC) (Czaczyk *et al*., 2007).

Several studies results confirm that microorganisms, being part of the biofilm, can be even 1000 times more resistant to the activity of toxic substances than those that remain in suspension. That makes their elimination from utility surfaces difficult (Fett, 2000; Trafny, 2000; Joseph *et al.*, 2001; Pancer *et al.*, 2004; Robbins *et al*., 2005; Pan *et al.*, 2006).

Microbial cells living in clusters form multilevel defense mechanisms against the destructive impact of antimicrobial substances. The nature of the structure of biological films causes slower diffusion of antimicrobial agents through the biofilm matrix composed of polymeric substances such as extracellular polymeric substances (EPS) or proteins and nucleic acids. It also makes it difficult for toxic agents to reach the deeper layers cells in a biofilm (Czaczyk *et al.*, 2007). Resistance is related as well to the specificity of metabolic and genetic changes induced during the growth phase of biofilm co-forming cells. These are mainly growth velocity decreases and biosynthesis of extracellular polysaccharides, enzymatic proteins and formation of proteins known as efflux pumps (Ma *et al.*, 1996; Nikaido, 1996; Berthold, 2007). The chemical communication of cells, called *quorum sensing*, based on the production of extracellular signal molecules in adverse environmental conditions, is also classified to the defense mechanisms of biofilm microorganisms (Golovlev, 2002).

The essence of the research reported herein was to confirm the assumption that the resistance of microorganisms to disinfecting agents is the outcome of their biofilm formation ability. To accomplish this, the bactericidal efficacy of disinfectant, commonly used for disinfection of equipment in the food industry was tested against bacterial biofilms formed on the stainless steel surface.

Microorganisms chosen for experiments dominate among life microflora isolated from food contact surfaces after cleaning and disinfection process. This meant that they exhibit resistance to the disinfectants, and therefore probably have a strong ability to create biofilms. These microorganisms are considered to be non-pathogenic, although some instances of their isolation from opportunistic infections have been reported. They rank as microorganisms responsible for crucial food components changes, related with synthesis of food quality lowering products, and are often defined as SSO (specific spoilage organisms) (Nowak and Piątkiewicz, 2008).

Experimental

Material and Methods

Bacterial strains and growth conditions. The bacterial strains used were *Micrococcus luteus*, *Staphylococcus hominis*, *Pseudomonas putida*, *Listeria innocua*, isolated from food contact surfaces, after a cleaning and disinfection process in a factory without CIP (clean-in-place) system. Stock cultures kept at –25°C in 20% glycerol were spread on tryptic soy agar (TSA) and incubated for 22–24 h at 30±1°C. A single colony of each strain was grown in 100 ml tryptic soy broth (TSB) at 30°C for 48 h to obtain bacterial suspensions of cells at the end of the logarithmic phase, at a density of 10^6–10^7 cfu/ml.

Preparation of stainless steel coupons. Stainless steel type 304 with number 4 finish was used to prepare coupons (5×5×0.1 cm). Coupons were cleaned with acetone to remove grease and were etched by submering in 5N HCl for 15 min and then cleaned in detergent solution The coupons were rinsed with deionized water, allowed to dry at room temperature, and then autoclaved at 121°C for 15 minut (Joseph *et al*., 2001).

Bacterial biofilm analysis. The sterile coupons were placed in sterile Petri dishes containing 2 ml bacterial suspension in TSB and 18 ml low nutrient medium TSB diluted ten times. After incubation at 20°C for 48 h, the samples were aseptically removed, washed in sterile phosphate buffer saline (PBS) to remove unattached cells and placed in Petri dishes with fresh sterile TSB. This procedure was repeated three times every 48 hours to complete the biofilm formation. To enumerate biofilm cells after eight days of incubation, the samples were washed with sterile PBS and the biofilm cells were removed by swabbing with sterile cotton swabs. The swabs were transferred to 100 ml 0.85% physiological saline peptone water, shaken vigorously and enumerated by standard spread plate technique. TSA was used for enumeration and plates were incubated at 30°C for 48–72 h. The bacterial biofilms on stainless steel were also recorded

Table I
Reduction of planctonic cells after 10 minutes exposure to usable concentrations of sanitizer.

Sanitizer concentration (%)	Contact time (min)	Mean population (log cfu/ml) and reduction (log cycles) of bacteria							
		Micrococcus luteus		*Pseudomonas putida*		*Staphylococcus hominis*		*Listeria innocua*	
		Population	Reduction	Population	Reduction	Population	Reduction	Population	Reduction
0,5%	10	8,15±0,19	6,04±0,29	8,00±0,06	7,38±0,35	8,03±0,11	6,34±0,52	8,04±0,13	6,07±0,41
1%			7,36±0,27		ND[a]		7,52±0,49		7,32±0,48

There are means and the standard errors of the means of triplicate in the table.
[a] ND no colonies detected in undiluted samples

by using scanning electron microscope 3000 N Hitachi. The stainless steel coupons (1×1×0.1 cm) with the biofilms before analysis were covered with a thin layer of gold.

Disinfectant agent. The disinfectant agent containing hydrogen peroxide and peroxyacetic acid was designed for use in the food industry. The active ingredients were: 25–30% hydrogen peroxide, 2–5% peroxyacetic acid, and 5–10% octanoid acid. The useable concentration of disinfecting agent was 0.5–1%. The sanitizer was diluted to the required concentrations with sterile deionized water. In the experiment the disinfecting agent was used in concentration 0.5%, 1%, 1.5%, 2% and the contact time for testing the sensitivity of biofilm cells was 10 and 30 min. The effectiveness of sanitizer to planctonic cells was tested in concentration 0.5% or 0.1% for 10 min.

Treatment of planctonic cells with disinfectant agent. Bacterial test suspension at a density from 1.0×10^8 cfu/ml to 5.0×10^8 cfu/ml was added to each tube containing 9 ml of disinfecting agent in concentration 0.5% or 1%. A timer was set and the contents were mixed in a microshaker. After 10 minutes the tested mixture was transferred to a membrane filter apparatus, which contained a membrane filter (∅ 0.45 mm) and 50 ml PBS and the whole was filtered. The filter was washed with 300ml PBS and transfered into Petri dishes with TSA. The number of cfu/ml was calculated after incubation at 37°C for 24–48 h. The decrease of bacteria count was calculated from the formula [log (N/N_a)], where N is the initial count of cfu/ml prior the treatment and N_a is the cfu/ml after treatment with disinfecting agent.

Treatment of biofilms with disinfectant agent. To test the sensitivity of biofilm cells to disinfecting agent, samples with biofilm were dipped in disinfecting agent solutions in the concentration of 0.5%, 1%, 1.5% and 2% for the contact time 10 and 30 min. The samples were then removed and rinsed with sterile PBS. The cells were enumerated after swabbing as described above using 0.85% physiological saline peptone water containing 3% Tween 80 and 0.3% lecitin and plating on TSA. Plates were incubated at 30°C for 48–72 h. The resistance of the biofilm to sanitizer was measured by a decrease in log values [log (N/N_a)].

Results

Bacterial adhesion to stainless steel. Microorganisms used in the examination showed biofilm formation ability on stainless steel (type 304, no 4 finish). After 8 days of incubation 20°C a thick multilayer biological film on the total plate surface was formed by *M. luteus* and *P. putida* (Fig. 1A, 1B), whereas *S. homins* and *L. innocua* formed biofilm, with a tendency to colonize the current irregularities and fissures on the steel surface (Fig 1C, 1D).

Effectiveness of sanitizer against planctonic cells. The test product in usable concentrations, 0.5%, for 10 minutes resulted in a reduction of viable cells of *L. innocua*, *S. hominis* and *M. luteus* by nearly 6 log cycles and *P. putida* by more than 7 log cycles. An increase of agent concentration to 1% for the same contact time caused complete inactivation of *P. putida*, whereas the count of other microbial cells was reduced by 7 log cycles (Table I). At higher concentrations or longer contact time an overall reduction in test cell suspensions of all tested microorganisms was observed (results not shown)

Effectiveness of sanitizer to biofilms. The cell density in the biofilm before and after the application of disinfecting agent is shown in following diagrams (Fig. 2). The average density of biological films formed by the investigated microorganisms reached appropriately 6.68 log cfu/cm^2 – *M. luteus*; 6.56 log cfu/cm^2 – *P. putida*; 6.13 log cfu/cm^2 – *S. hominis*; 5.87 log cfu/cm^2 – *L. innocu,* after 8 days of incubation at 20°C. Application of disinfecting specimen in operational concentration of 0.5% for 10 minutes showed low effectiveness on the biofilms formed by all the bacteria tested and caused the reduction in the number of cells by only about 1–2 log cycles. After 30 min contact time reduction of 3–4 log cycles was observed. Use of the highest concentration recommended by the producer, 1% in 30 minutes, resulted in a reduction in the number of cells by 5 logarithmic cycles for only one organism – *M. luteus*. For other types of bacteria the reduction rate of cells was about 3 to 4 log cycles. When the concentration of applied substance increases, with time of contact 10 minutes, a gradual increase in the reduction of microorganisms

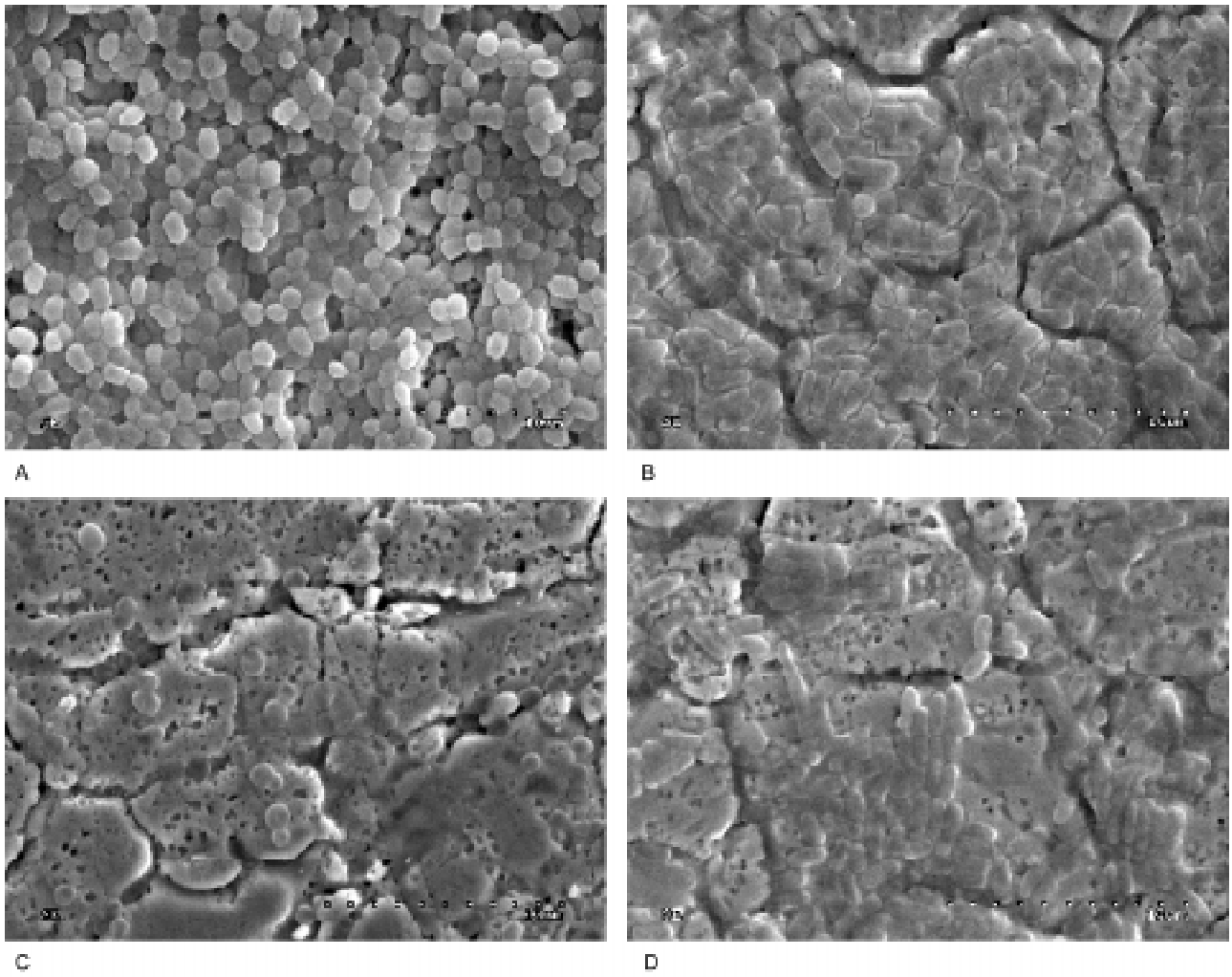

Fig. 1. SEM microphotographs of biofilm: A – *Micrococcus luteus*, B – *Pseudomonas putida*, C– *Staphylococcus hominis*, D – *Listeria innocua* formed on stainless steel for 7 days at 20°C.

in biofilms was observed, although the reduction range was not higher than 5 log. Extending the contact time to 30 minutes resulted in a drop in survival of log cycles. As shown in the figures, only increasing the concentration of the agent twofold and extending the time of contact to 30 minutes brought about a reduction of over 5 log cycles for all the studied microorganisms. The study also indicated that *M. luteus* had a much lower resistance than the other bacteria used in the study – its survival was approximately 1–2 log lower compared to the other species.

Discussion

The purpose of this research was to estimate the antiseptic efficiency of disinfecting agent towards to bacterial biofilms formed on stainless steel. Model studies were carried out on biofilms created in laboratory conditions that simulate the food processing environment. Therefore, the formation of biofilms was carried out in stationary culture for 8 days, oligotrophic conditions of growth were applied and incubation was at 20°C. The objective of this study was to confirm the thesis that the microflora living on production surfaces are resistant to disinfectants, in spite of correctly performed washing and disinfection procedures, and at the base of this resistance is the ability of the microorganisms to form a biofilm.

Research was carried out using microorganisms defined as conditionally pathogenic, which dominated among microflora isolated after cleaning and disinfection. This proves their resistance to applied disinfectants as a possible result of biofilm-forming ability. The experiments carried out by Krogulska (2003) indicate that this group of microorganisms colonizes solid surfaces the most effectively. Moreover, the presence of these microorganisms on the food contact surfaces, determines the deterioration in the quality of food products.

Bacterial biofilm formation on abiotical surfaces is a persistent problem in the food industry. Disadvantageous conditions that prevail after cleaning and disinfection process favor the formation of biological

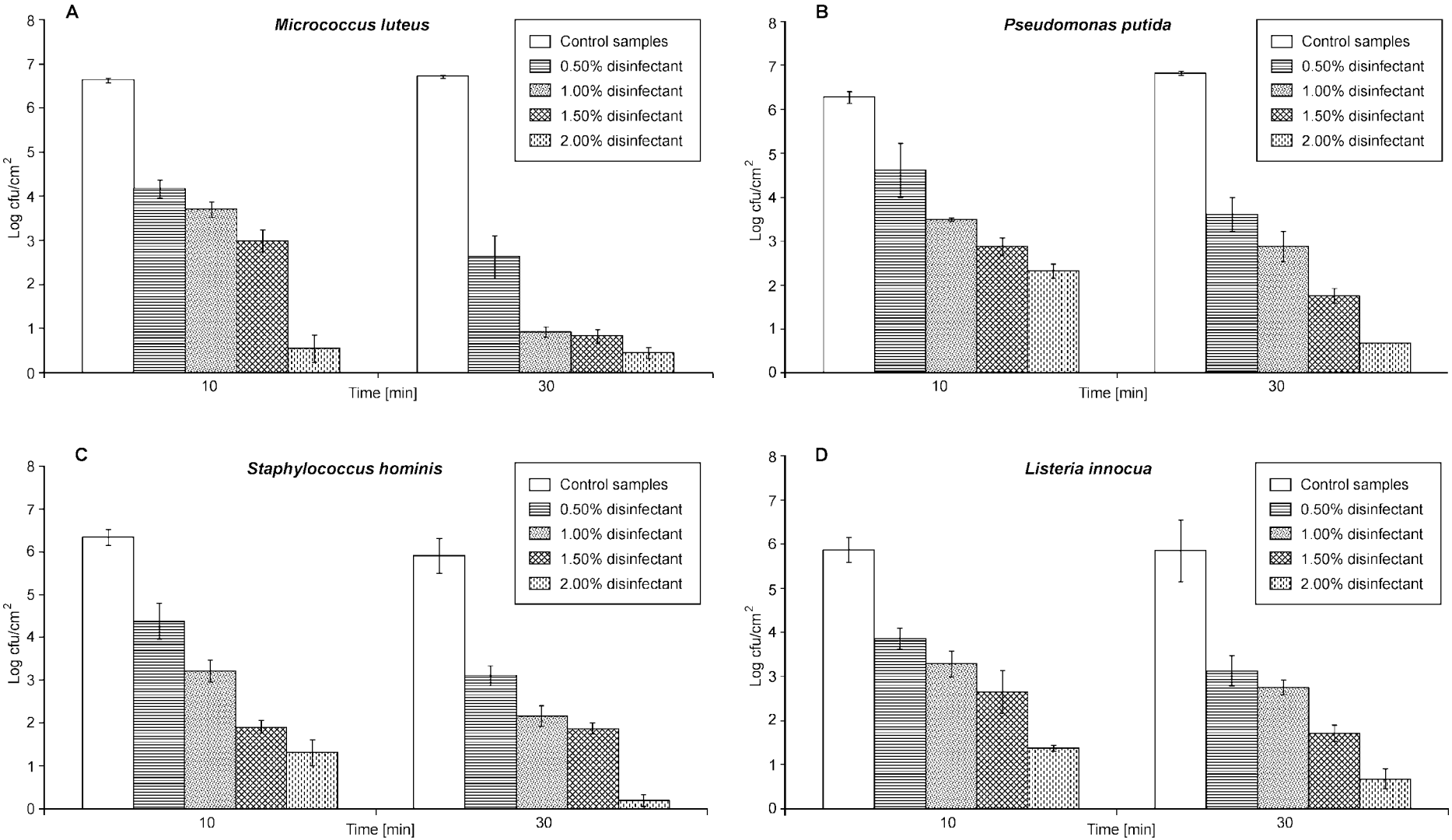

Fig. 2. Effect of tested disinfectant on biofilm formed by *Micrococcus luteus* (A), *Pseudomonas putida* (B), *Staphylococcus hominis* (C) and *Listeria innocua* (D), depending on concentration and contact time.

The white column represents control samples without sanitizer treatment. The colored columns represent samples after sanitizer treatment. The error bars indicate standard deviation.

films. Several studies results prove that the adhesion of bacterial cells to abiotic surfaces is more intensive in hunger conditions. Morphological and physiological changes such as metabolic activity decrease or excessive secretion of extracellular molecules are major determinants of this phenomenon (Fleming and Wingender, 2001; Folsom *et al.,* 2006).

Food production in terms of microbiological safety requires effective eradication of biofilms formed by microorganisms appearing naturally in various ecosystems. The studies conducted as well as several literature sources show that the inactivation of microorganisms in biological films of usable materials is not effective enough (Lee Wong, 1998; Chmielewski and Frank, 2003; Midelet and Carpentier, 2004; Ingham, 2006). Difficulties in elimination of microbiological biofilms from solid surfaces mainly due to ignorance of the characteristics determining the resistance of microorganisms belonging to different taxonomic groups. The result is that the commonly available disinfectants are not suitable for inactivation of biofilm.

Properly carried out disinfection should result in the reduction of microorganisms by at least 5 logarithmic cycles (EN 1040, 2006; EN 1276, 2000; Borycki *et al.*, 2008). The test disinfectant in useable concentration 0.5%, and 10 minutes reduced the number of cells in the biofilm by about 1–2 logarithmic cycles. Only after doubling the concentration to 2%, with a long-time contact of 30 minutes, the disinfectant met the requirements specified in the standard in relation to the cells in the biofilm. Bacteria in suspension were effectively reduced at a concentration of 0.5%, for 10 minutes.

Results of realized experiments showed that microorganisms staying on production surfaces are able to formation of biofilms and they are more resistant to disinfecting agents than planctonic cells. This was also confirmed by other authors. Joseph *et al*. (2001) investigated *Salmonella* spp. biofilms sensitiveness formed on various surfaces. They demonstrated that fivefold active chlorine concentration and twice longer time is needed for *Salmonella* spp. biofilm inactivation of 6 log cfu/ml cells density on stainless steel than for the complete cells reduction in equal density suspension. Similarly, the experiments carried out by Robbins *et al*. (2001), have shown that the effective inactivation of *Listeria monocytogenes* cells in a biofilm requires the use of a twice higher concentration of hydrogen peroxide, than to inactivate planctonic cells.

The high resistance of cells in the biofilm is being explained, among other things, by slower diffusion of antimicrobial agents through the biofilm matrix, which make it difficult to reach the deeper biofilm layers. The rate of diffusion of chemical substances through the biofilm layers can be even 60–80% slower. Moreover, cells that stay in the suspension are exposed to toxic substances on all sides and cells in the biofilm only from one direction (Myszka and Czaczyk, 2007).

To elaborate effective strategy of biofilms removal from food contact surfaces, it is essential to get to know adhesive properties and resistance factors of microorganisms that live in various environment conditions. It is well known that the crucial role in formation of biofilm resistance to antimicrobial specimens is played by the EPS protective layer formed by the colonizing cells. Research carried out by Szumigaj *et al*. (2008) and by Czaczyk *et al.* (2004) showed that excessive secretion of extracellular molecules takes place when the access of nutrients is limited. These extracellular molecules, as a result of solid surface adsorption, mediate in the cohesion and adhesion of microorganisms. Exopolysaccharides are also the main component of a highly hydrated glycocalyx layer that enables to immobilize the microcolony of cells and protects them against biocides and other antimicrobial substances negative effect (Costerton *et al*., 1995).

Three among tested microorganisms (*L. innocua, S. hominis*, *P. putida*) had similar resistance ability to disinfectant used in experiments. Thus, it is advisable to assume that bacteria, adapted to environmental conditions, which have been isolated, have developed similar mechanisms of resistance.

Among all tested microorganisms, *M. luteus* showed the lowest resistance to the specimen compared to other microorganisms. The reason may be due to different properties of the bacterial cells themselves, but also creating the monoculture biofilm during experiments. Biological films that are formed in natural environment conditions, can be composed of one to several species and their functioning is based on particular microorganisms interaction. Metabolites of single species of microorganism can stimulate the growth of other biofilm matrix co-forming cells (Czaczyk, 2004; Dunne, 2002). Studies by Burmolle *et al.* (2006) proved that the synergistic interactions that occur in non-homogeneous biofilms can bring about an increase in their resistance to antimicrobial agents in comparison with homogenous biofilms. It can be assumed, that in the natural environment, the resistance of *M. luteus* could be further enhanced by the presence of other microorganisms, which would explain such frequent isolation of this microorganism from the production surfaces.

The results of our experiments indicate that the tested disinfectant has eradicating activity towards to planctonic cells as well as cells in a biofilm. However, it must be stated that biofilms are characterized by much higher resistance than cells in suspension and they require the application of disinfectants in higher concentrations. Therefore, hygienic procedures performed in the factories, with the use of chemicals should also take biofilms into account.

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