2

Antimicrobial Activity of Quaternary Ammonium Salts and Evaluation of Research Methods

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1. Introduction

Since Poland has become EU member, the quality of raw materials and food products have became a priority in food industry plants. The law act of EU concerning the food is Directive 93/43/EEC from June 14, 1993 with reference to hygiene of food products. From a health protection point of view the main problem are poisonings and infections resulting from pathogenic microbes developed in the food products. Besides the microbial purity of raw materials, the washing and disinfection lines in food processing plants are a very important preventive measures [Waszak 2004; Żakowska & Stobińska 2000].

In chemical disinfection of machines and facilities in a food processing industry many preparations have found an application, from about 65% made up substances containing quaternary ammonium salts (QAS). Their action may be divided into two stages. In the first, a change in permeability of microbial cell-membrane occurs, which results in deterioration of a proper exchange of nutrient elements with environment. In a second stage it comes to a denaturation of enzymes in a cytoplasm membrane and to disturbances of a whole cell metabolism [Waszak 2004; Wiśniewski 1997].

The assortment of these preparations is different and considerable, which is a result of their chemical structure $CR_1R_2R_3R_4X$ where as the organic substituents may be both alkyl chains and aromatic nuclei and as halogen ion may be often chloride. Additionally there are many possibilities to mix the quaternary ammonium salts with different auxiliary substances in order to increase their effectiveness and to increase the number of possible combinations [Steinka & Przybyłowski 1999; Żakowska & Stobińska, 2000].

The advantages of these preparations are: a high biocidial efficiency (they are effective against gram-positive and gram-negative bacteria, yeast, some mycelium fungi and viruses), a prolonged effect of microbial purity, low toxicity as well as a mild and pleasant smell. The QAS lower a surface tension and

effectively penetrate a dirt layer and do not cause a corrosive action on disinfected surface. They work by 4-10 pH, have a good solubility in water and a high durability in concentrated and working solutions, which is their additional advantage [Steinka & Przybyłowski 1999; Żakowska & Stobińska, 2000].

The disadvantage of these preparations is a foaming ability, which makes it difficult to rinse the washed items. Hard water, organic impurities e.g. proteins, fats, blood and a presence of soaps decrease their antimicrobial activity [Wiśniewski 1997]. In order to eliminate the danger of microbial resistance, it is recommended to use them alternately with preparations with other mode of action.

2. Aim and Scope of the Investigations

The aim of undertaken investigations was to evaluate and compare an antimicrobial activity of QAS against most frequently occurring microorganisms in a food industry. The investigations were carried out in laboratory conditions with the methods recommended by NIH.

3. Material and Methods

A choice of the desinfectants used was made on the basis of a chemical structure of QAS. The active substances of the preparations were:

A – 10% of didecylodimethylammonium chloride, phosphonates, nonione tensides, smell substances, B – 10% didecildimethylammonium chloride, gluconate chlorhexydyne, dihydroxyethylalkylamine oxide, C – 1% of didecyldidimethylammonium, 55% by weight of isopropyl alcohol.

In the investigation working solutions were used made according to the producer recommendations: A- 5%, B – 1%, C – undiluted.

Bacteria and fungi under investigations were taken by isolation from food products and belonged to the strains stored in a Microbiology research laboratory. For the tests used 0.5% suspensions (according to Mc Farland scale) of *Escherichia coli, Bacillus subtilis, Bacillus cereus, Aspergillus sp., Penicillium sp., Fusarium sp.*. The bacterial culture was carried out on nutritional agar (24 h – 37^{o}C) and fungi culture on Sabourad breeding medium (5days- 22^{o}C). As a control a deionized, sterile water was used.

Antimicrobial activity of chosen disinfectants was evaluated according to by NIH recommended techniques:

The disc-diffusion method (bacteria, fungi) – the suspensions of bacteria or fungi were distributed on an adequate medium. Then disc tissue paper impregnated with disinfectant was put on a plate. As an index of sensitivity of

the microbial cultures, a size of inhibition zone was taken [Krzywicka *et al.*, 1982].

The carrier (stamp) method (bacteria, fungi) - a metal stamp covered with a sterile fabric was infected by a microbe suspension and then remained for drying up at an ambient temperature. The dried stamp was then treated with a disinfectant, rinsed in distilled water and after drying up, a microbial suspension was distributed. The number of microbial colonies after this procedure was accepted as a basis of evaluation [Krzywicka *et al.*, 1993].

The flood method (bacteria) – a disinfecting solution was at first mixed with a bacteria suspension and then neutralized. Using this flood method of Koch it was possible to evaluate the reduction degree of the number of tested microorganisms after time of contact with the disinfectant [Krzywicka *et al.*, 1982].

The method of a reversed diffusion (fungi) – the susceptibility of fungi involved observation of a mycelium development on adequate substratum contained disinfectant. The size of mycelium development was the basis of an evaluation [Krzywicka *et al.*, 1982].

4. Results

Statistic analyses of results using three different methods proved that tested microorganisms significantly differed from each other taking into account reaction for disinfectants, based on quaternary ammonium salts, prepared according to producer directions. Interaction significance between analysed factors was also proved.

The methods used for an evaluation of a antimicrobial activity of disinfectants showed a great uniformity of results. Values of correlation coefficient were as followed:

- r = 0,956**, where methods based on evaluation of number of microbial colonies (stamp and flood Koch's method) were compared,
- r = -0,958** and -0,872** for comparing methods based on different evaluation criterions. It is: evaluation of number of microbial colonies (stamp and flood Koch's method) and evaluation of size of inhibition zone (disc-diffusion method). In this case the minus value of correlation coefficient means that the stronger inhibition of colonies number on plate caused by tested preparations, the higher growing inhibition by disinfectants.

Despite of correspondence of achieved results, using methods with different precision characterised bacterial reaction for used disinfectants. The stronger bacterial reaction was characterized by flood Koch's method. It was expressed by the highest value of variability coefficient (V=78,2%) and the

wider range of evaluation values. Expressing it as a deviation from arithmetic mean (0%) it was range from -99,9 to + 127,2% (Σ 227,1%). Analogical for other methods it was for:

- stamp method: V=65,9%, range from -93,8 to +85,8% (Σ 179,6%),
- disc-diffusion method: V=50,6%, range from -57,2 to +84,9% (Σ 140,1%).

Independently of kind of preparations the lowest sensitivity evaluated by above methods had bacteria species of *Bacillus subtilis* (tab. 1). It was characterised by the lowest changeability to analysed factors (V=105%) and the highest range of evaluation range from -95,8 to +167,9% (Σ 265.8%) of deviation from arithmetic mean. In the case of *Bacillus cereus*, which occurred as a the most sensitive, reaction changeability and ranges were the highest and amounted appropriate V=127,4%; range from -99.9 to +327.3% (Σ 427,2%) of deviation from arithmetic mean.

Correspondence of reaction of above bacteria species as well as *B.cereus* and *E.coli* for used disinfectant was proved by statistic. It means together with reaction significance bacteria species x disinfectants that their reaction was shaped different and it depended on preparation kind. The same significant results showed *B. subtilis* and *E.coli* (r=0,857**). Nevertheless bacteria growth, especially of *E.coli*, was limited the strongest by preparation consist of didecylodimethylamonium chloride and isopropyl alcohol.

Fungi's sensitivity evaluated by different methods was significantly correspondent in all cases with reaction of tested bacteria species. Correlation coefficients were in range from 0,696 – for comparing of *Penicillium* sp. Reaction with *B.subtilis*, to 0,966** in the case of *B.cereus*.

Comparing to bacteria fungi reaction for tested preparation was more diverse, especially in disc-diffusion method. It was expressed by the highest value of changeability coefficient (V=90.1%) and the wider range of evaluation values. Expressing it as a deviation from arithmetic mean (0%) it was range from -99,6 to + 123,9% (Σ 223,6%). Analogical for other methods it was for:

- stamp method: V=81,2%, range from –84,4 to +118,1% (Σ 202,5%),
- disc-diffusion method: V=66,4%, range from –77,8 to + 97,0% (Σ 174,8%).

Table 1. Disinfectants effectiveness against bacteria and fungi evaluated with four methods

Tabela 1. Skuteczność dezynfektantów oceniona czterema metodami

Methods	Microorganisms tested	Disinfecants			
		A (solution 5%)	**B (solution 1%)**	**C (nondiluted)**	**H_2O (control)**
Disk – diffusion method (inhibition zone in cm)	**Escherichia coli**	5.33	5,83	7,00	0,00
	Bacillus subtilis	5,08	3,25	4,17	0,00
	Bacillus cereus	13,00	8,25	13,42	0,00
	Aspergillus sp.	2,00	1,00	6,00	0,00
	Penicillium sp.	2,92	0,50	5,00	0,00
	Fusarium sp.	0,68	0,00	6,00	0,00
Germ carrier method (no of colonies)	**Escherichia coli**	17600	120,67	81,83	500,00
	Bacillus subtilis	158,33	208,33	176,67	500,00
	Bacillus cereus	11,33	69,00	7,00	120,00
	Aspergillus sp.	120,00	140,00	16,00	200,00
	Penicillium sp.	20,00	150,00	15,00	200,00
	Fusarium sp.	150,00	200,00	14,33	200,00
Flood method (no of colonies)	**Escherichia coli**	45,67	28,33	8,33	120,00
	Bacillus subtilis	30,33	61,67	46,67	200,00
	Bacillus cereus	4,33	19,00	0,00	100,00
Reverse diffusion method (size of colonies)	**Aspergillus sp.**	12,00	14,00	2,42	18,92
	Penicillium sp.	4,33	13,08	3,17	18,25
	Fusarium sp.	12,33	17,75	2,00	22,50

Similar like at bacteria, used methods at significant correspondent way characterised fungi reaction for tested disinfectants. Values of correlation coefficients were as followed:

- r = 0,989**, where methods based on evaluation of number of microbial colonies (stamp) and its size (reserved diffusion) were compared,
- r = -0,929** and -0,944** for comparing method based on evaluation of number of colonies (stamp) and its size (differ diffusion) with method of size evaluation of inhibition zone (disc-diffusion method). The minus value of correlation coefficient means that the lower number and size of fungi colonies on plate, the higher growing inhibition by tested disinfectants.

Independently of kind of preparations the lowest sensitivity evaluated by above methods had fungi of *Fusarium* sp. (tab. 1). Changeability of its reaction to analysed factors was at a range of 167,5% and the range of evaluation values was from -99,9 to +346,5% (Σ 446,4%) of deviation from arithmetic mean. Fungi *Penicillium* sp. was the most sensitive, where the method of carrier stamp and reversed diffusion was used. Changeability of its reaction and a range were the highest and amounted appropriately: V=628,7%; range from -97,9 to +530,8% (Σ 628.7%) of deviation from arithmetic mean. Nevertheless, in case of disc-diffusion method, a little bit more sensitive occurred fungi *Aspergillus* sp., with the lowest range of changeability (V=156.4%) and the more narrow range of evaluation values (from -97,1 to +302,0%; Σ 399,1% of deviation from arithmetic mean).

Despite of differences, reaction of tested fungi's to above disinfectants was significantly correspondent, and values of correlation coefficient were from r=0,997 to r=0,791*. The highest sensitivity reaction of tested fungi was observed with preparation consist of didecylodimethylamonium chloride and isopropyl alcohol.

5. Discussion

The evaluation based on four methods from which the two were the universal ones (the disc-diffusion and carrier method) and were suitable both for bacteria and fungi and the remaining two (the flood and of reversed diffusion methods) which may be defined as the target methods. The methods differed first of all by a way of their performing. The most quick and easy to perform were the disc-diffusion and reversed diffusion methods. The low contamination probability from environment is an additional factor in favor of their application. The most difficult performing procedure presented the carrier (stamp) method. The long performing time as well as the possibility of a material contamination in case of low level of sterility in a lab may lead to false evaluation results.

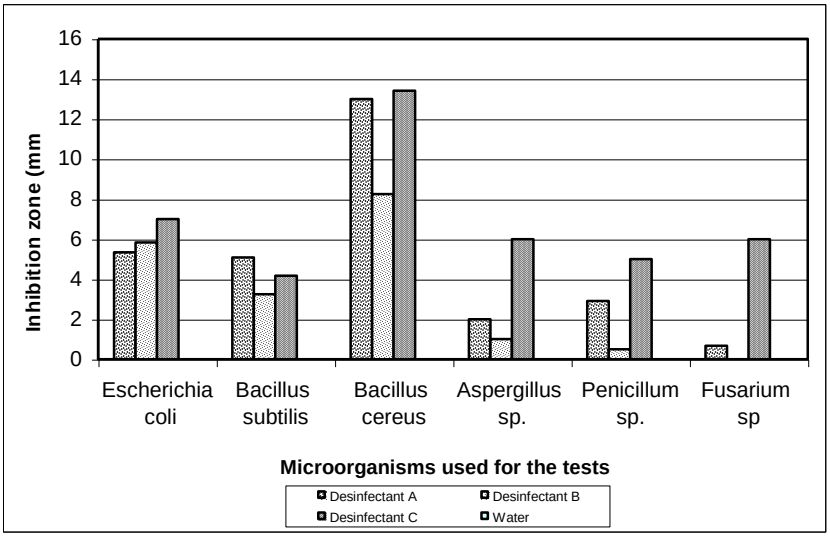

Fig. 1. Size of inhibition zone in disc – diffusion method
Rys. 1. Rozmiar strefy inhibicji – metoda dyfuzji

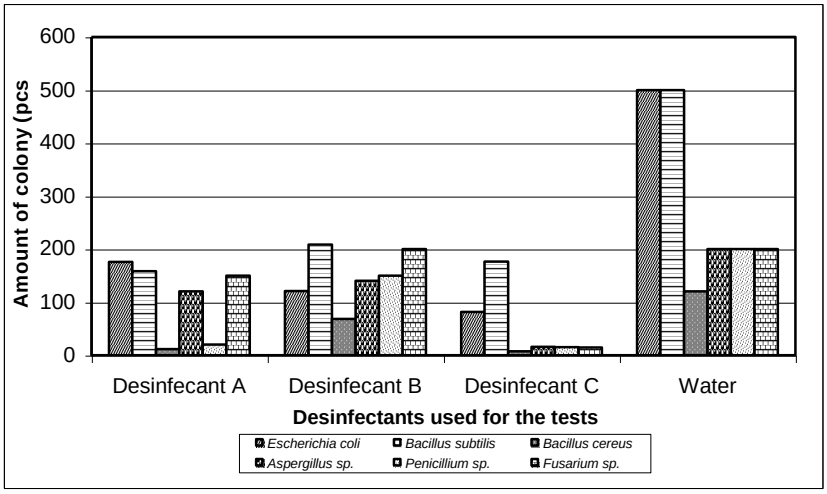

Fig. 2. Amount of microbes colonies in germ carrier method
Rys. 2. Ilość kolonii bakterii w metodzie nosiciela bakterii

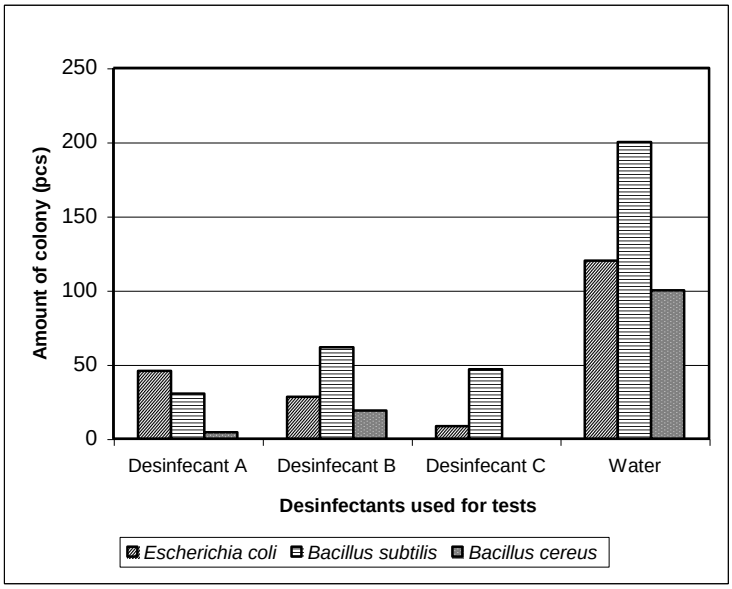

Fig. 3. Amount of bacteria colonies in flood
Rys. 3. Ilość kolonii bakterii w zalewie

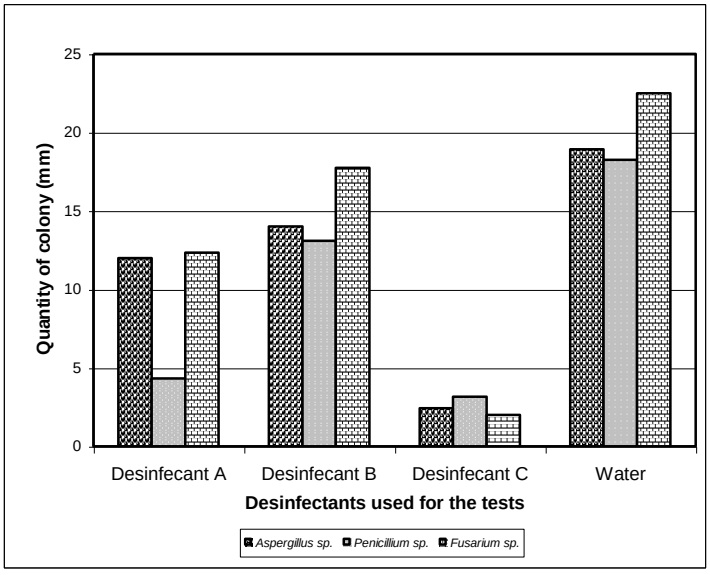

Fig. 4. Amount of fungus colonies in method reverse diffusion method
Rys. 4. Ilość kolonii grzybów w metodzie odwróconej dyfuzji

The important feature of the used methods is their sensitivity. In this relation the best in a case of bacteria was a flood method and in a case of fungi the method of reversed diffusion.

The differentiated biocidial efficiency of the evaluated disinfectants resulted mainly from a different concentration of QAS applied in working solutions (A-0,02%, B- 0,5%, C- 1%) and from additional substances contained in these disinfectants. So, it may be supposed that the highest activity of the disinfectant C against bacteria and fungi resulted from its chemical composition, where the active ingredients were both QAS (1%) and isopropyl alcohol (55% by weight). The working dissimilarity of two remaining preparations resulted mainly from their different chemical composition. The disinfectant A besides QAS contained also phosphonates (softening substances) and nonione tensides (surface active substances). The main task of these substances is to decrease surface tension between two contacting phases and to ensure good penetration of active substances into a cell inside. So, this preparation proved to be better than the preparation B, despite of a lower concentration of QAS.

The different susceptibility of bacteria on the preparations used in this work resulted first of all from heterogenous structure of cell walls. *Escherichia coli* belongs to the gram-negative bacteria, so a permeability of its cell walls is more poor than that of a genera *Bacillus* (gram-positive). In support of this statement may be used the results received for *Escherichia coli*, the reaction thereof on the used disinfectants was most differentiated. The next reason for the resistance against the used disinfectants was the bacteria ability to a biodegradation of these substances. *Bacillus subtilis* which was most resistant eliminates for instance a nitro group from derivative of phenols which is an ingredient of these compounds.

The resistance of fungi resulted also from a specific structure of their cell walls. The cell wall of fungi is more thick and rigid than that of bacteria and is composed from chitin, glucan, lipids and proteins what make the process of permeability to the cell inside more difficult. In a case of *Fusarium sp.* the resistance against disinfection was accompanied with a better nitrogen assimilation which decreased the biocidial activity of QAS.

The main task of a disinfection of machines and facilities in a food processing industry is a destruction of microorganisms, in order to prevent their transmission to food. The above presented results show that the hygienic procedure does not destroy completely the occurring microflora. It should be underlined that the evaluations were carried out in laboratory conditions where the resistance of microorganisms on the disinfectants is much weaker than that in a nature. At present the main problem is with bacteria attached to solid surfaces in a form of a biolayer and the resistance thereof may be 500-fold greater, when compared with free-living cells [Królasik, 11/05]. The washing and disinfecting

procedure in food processing plants should therefore be careful and regular following the accurate cleaning the surfaces from organic residues.

6. Conclusions

1. The highest antibacterial and antifungal activity has shown the preparation containing 1% of quaternary ammonium salts and isopropyl alcohol as an auxiliary substance.
2. The using of the preparations on a basis of quaternary ammonium salts does not assure the expected microbial purity on surfaces of machines and process engineering facilities.
3. Independently of a producer recommendation it would be advisable to evaluate the effectiveness of disinfectants in industrial conditions where they will be used.
4. From the methods being applied the most easy to perform was the disc-diffusion and most difficult in this relation was the carrier (stamp) method.

References

1. **Drożdż I., Tuszyński T.:** *Substancje szkodliwe i toksyczne w żywności i napojach.* Laboratorium *3*/06 s. 52÷62.
2. **Gnaciński P.:** *Czyste ręce – zdrowia więcej.* Bezpieczeństwo i Higiena Żywności, 3\14\2004, s. 28÷33.
3. **Królasik J.:** *Biofilm - mikrobiologiczna strategia przetrwania.* Przegląd Piekarski i Cukierniczy 11/2005, s. 14÷18.
4. **Krzywicka H., Janowska J., Tadeusiak B., Zarzycka E.:** *Metoda określania stężeń użytkowych preparatów dezynfekcyjnych – Metoda Nośnikowa*. Wydawnictwa Metodyczne PZH 1993.
5. **Krzywicka H., Bielicka A., Janowska J., Jaszczuk E., Tadeusiak B.:** *Metody badania aktywności bakteriobójczej preparatów dezynfekcyjnych.* Wydawnictwa Metodyczne PZH, 1982, s. 33÷38.
6. **Steinka I., Przybyłowski P.:** *Podstawy mikrobiologicznej analizy żywności.* Ćwiczenia, Wyższa Szkoła Morska, Gdynia 1999, s. 107.
7. **Waszak B.:** *Wpływ dezynfekcji na wysoki poziom czystości mikrobiologicznej.* Zakażenia 4/04, s. 20÷23.
8. **Windyga B., Grochowska A., Urbanek-Karłowska B., Napiórkowska B.:** *Preparaty dezynfekujące dopuszczone do stosowania w zakładach przetwórstwa spożywczego.* Przemysł Spożywczy, 6\96, s. 27÷30.
9. **Wiśniewski W.:** *Preparaty dezynfekcyjne zakres stosowania zalety, wady.* (Część 1, 2). Przemysł fermentacyjny i owocowo-warzywny, 6\97, s. 18; 7/97, s. 32÷35
10. **Żakowska Z., Stobińska H.:** *Mikrobiologia i higiena w przemyśle spożywczym*. Wydawnictwo Politechniki Łódzkiej. Łódź 2000. s. 40, 62-65, 106-111, 237, 275, 309-315, 489.