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INVESTIGATION OF ACTION OF PEROXOACETIC ACID ON LIPID COMPONENT OF BACTERIAL SPORES AND CONTRIBUTION TO THE STANDARDIZATION OF EFFICIENCY EVALUATION TEST

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The aim of this research was to contribute to a more detailed insight in the action of peroxygen disinfectants based on peroxoacetic acid (PAA), on lipid components of sporogenic forms of microorganisms. Sporogenic forms are recognized to be significantly more resistant in comparison to vegetative forms. Bacillus cereus ATCC 11778 and Bacillus subtilis NCTC 10480 where chosen as referent bacterial sporogenic strains used to investigate the acting mechanism of PAA. After treatment of the above mentioned bacterial strains with PAA, fatty acids have been isolated from untreated and treated spores and consequent changes in the lipid component were analyzed. The obtained methyl-esters of fatty acids (MEFA) were analyzed by gas chromatography/mass spectrometry (GC/MS), using a standard of bacterial MEFA. Results confirmed that after treatment, in Bacillus cereus certain quantitative changes occurred, which included the decrease in quantity of fatty acids with 16 and 17 carbon atoms, i.e. 16:0, 16:1, iso 17:0 and anteiso 17:0. At the same time, in B. Subtilis the significant decrease in quantity of fatty acids with 15 and 17 carbon atoms occurred, i.e. anteiso 15:0 and anteiso 17:0.

Key words: bacterial spores, fatty acids, lipid component, peroxoacetic acid, peroxygen disinfectants

INTRODUCTION

The importance of proper sanitation deserves a prominent place in public health protection. Special attention should be given to sporogenic bacterial strains. The higher rate of resistance of spores to a wide range of chemical agents (acids, bases, phenols, aldehydes and oxidizing agents) in comparison with vegetative bacterial forms is confirmed (Sagripanti and Bonifacino,1999). *Bacillus cereus ATCC 11778* and *Bacillus subtilis NCTC 10480* where chosen as referent bacterial sporogenic strains which were used to investigate the acting mechanism of PAA (Heeg and Brill, 1998). In many cases the mechanism of resistance is not well known, although some data show that certain oxidizing agents may provoke

damage of protein components, while others, e.g. alkaline agents provoke the damage of cell DNA (Nicholson *et al*., 2000)

Peroxoacetic acid (PAA), whose sporicidal effect has been investigated, can be considered to be a more potent biocide than hydrogen peroxide or chlorine based agents, taking into consideration that it showed excellent sporicidal, bactericidal, virucidal and fungicidal properties even at low concentrations <0,3% (McDonnell and Russell, 1999).

In the environment, PAA is subject to decomposition to safe by-products like acetic acid and oxygen (McDonnell and Russell, 1999): $CH_3COO(O)H \rightarrow CH_3COOH + 1/2O_2$ and this is an additional advantage in comparison with hydrogen peroxide because PAA does not undergo the process of decomposition by the action of peroxidase and retains its activity even in the presence of organic challenge. It showed to be effective even at low ambiental temperatures and in the presence of hard water. It is also used as a liquid sterilant for medical equipment and instruments, in the food industry for disinfection of equipment and as a sterilant for environmental surfaces. In up to date investigations, the sporicidal effect of PAA on spores of *B. anthracis* has been confirmed, even in the presence of 4% of horse serum and at 20°C. The sporicidal effect was even 32 folds stronger when compared with glutaraldehyde and 64 folds stronger when compared with formaldehyde (Malchesky, 2001). Further on, comparison of PAA with other disinfectants, i.e. active chlorine and benzalconium-chloride, against food-poisoning bacteria also showed the supremacy of this biocide (Orth and Mrozeck, 1989).

The acting principle of PAA could be based, on the denaturation of proteins i.e. enzymes, by breaking sulphydril (-SH) and disulphyde (S-S) bonds, thus provoking increased permeability and consequent disruption of the cell membrane (McDonnell and Russell, 1999).

Available data on the acting principle of PAA on the lipid component of bacterial spores is insufficient. For the purpose of the evaluation of sporicidal effects of PAA and for the estimation of active concentrations, standardized tests for efficiency evaluation of biocides, approved in EU were applied (A. Cremieux *et al*., 2001).

- Basic bactericidal activity - EN 1040 (1997)
  Phase 1 suspension test
- Basic sporicidal activity - CEN/TC 216, EN 216003 (1998)
  Phase 1 suspension test

In order to perform the efficiency evaluation of the disinfectant "Germiper", in the presence of organic challenge (conditions similar to the environment) the following tests have been used:

- Application test for the field of veterinary medicine (bactericidal activity) – EN 1656 (1999)
  Phase 2 step 1 suspension test
- Application test for the food industry and public health institutions (bactericidal activity) – EN 1276 (1997)

MATERIALS AND METHODS

In experiments for the evaluation of the efficiency of peroxygen disinfectants (PAA) and extraction of fatty acids before and after treatment with PAA, we used bacterial strains *B. subtilis ATCC* 11778 and *B. cereus NCTC 10480.* For that purpose disinfectant "Germiper" (manufactured by: "NRK Inzenjering", Belgrade) has been used. This biocide is equilibrated solution of water, acetic acid, hydrogen peroxide and peroxoacetic acid. Before carring out the efficiency evaluation test, the chemical characterization of "Germiper" has been performed and afterwards its sporicidal effect was evaluated. Results of chemical characterization of two samples of "Germiper", in average, were as follows: 1.081 g/cm^3 – density, 0.87 – pH, 11.22% – active oxygen, 10.14% – PAA, 18.02% – hydrogen peroxide and 7.52 g/L – sulphates. In addition, pH values of tested active concentrations of Germiper were as follows: active concentration 0,5% at pH 3, active concentration 1% at pH 2.7, active concentration 2% at pH 2.5 and active concentration 4% at pH 1.5.

Phase 2 step 1 suspension test

The organic challenge included defibrinated horse blood (3 g/L). First step of suspension test was the preparation of test microorganisms culture suspension with initial number of 10^6 microorganisms in the inoculum. The homogenized suspension was prepared from 18h old culture (agar nutrient media). The evaluated active concentrations of "Germiper" were 0,5%, 1%, 2% and 4%, at 20^{o}C and in the presence of hard water (400 ppm). From the so prepared suspension we have taken 0.1 ml with a sterile pipette and transferred it into 10 ml of each of the four evaluated concentrations of disinfectant. After 5 min, 15 min, 30 min and 60 min we inoculated the so treated bacteria into buoyon nutrient media (10 ml) and incubated at 37^{o}C, for 24-48 hours. Finally, we checked for bacterial growth. The liquid nutrient media, inoculated with test cultures, was used as a control.

Biomass production

In order to obtain the needed biomass of *Bacillus cereus ATCC 11778* and *Bacillus subtilis NCTC 10480*, we performed bacterial fermentation in a liquid nutrient media (2x200 ml, in erlenmeyer flasks). After inoculation with bacterial strains, incubation was performed in a shaker (180 rev/min) at 37^{o}C, for 24 h. After completed fermentation we kept the bacterial suspension at 4^{o}C, for 7 days in order to obtain sporogenic forms which were used in the experiment. In order to exclude possible vegetative forms in the inoculum, we heated the aliquots at 80-85^{o}C, for 1-5 min after that we inoculated it on agar plates. In addition, we inoculated unheated samples on agar plates and compared the number of bacterial colonies with heated samples (Tanimoto *et al*., 1996). The starting material which we used for isolation of fatty acids was biomass in wet form which we were separated by the centrifugation (4000 rpm/15 min). Then we washed the obtained precipitate with physiological solution and centrifuged it as previously. After that we performed hydrolysis and methylation of obtained biomass and used

methyl-esters of fatty acids for GC/MS analysis. We also followed the same procedure for untreated (control) samples.

Treatment with PAA

In order to obtain the needed biomass 100 ml of bacterial suspension was centrifuged at 4000 rpm. The precipitate was washed with saline, then centrifuged and after that we added 1% solution of "Germiper" and exposed it to slow mixing movements for 30 min. After treatment with PAA the suspension was centrifuged at 4000 rpm/10 min and the supernatant was discarded. After rinsing with distilled water, we used the sediment for isolation of fatty acids in treated samples. The biomass after treatment with PAA and the control samples were treated in the manner followed in the next steps for isolation of fatty acids.

Hydrolysis and methylation of fatty acids

In a round bottom flask with reflux 400 mg of wet biomass in mixture of toluol:methanol:sulphuric acid – 10:10:0.4 [v/v/v] was heated in a hot wather bath for 3 h. After cooling, 10 ml of saturated solution of NaCl was added and then we performed the extraction with a mixture of chloroform: hexane – 1:4 [v/v] (2x15 ml). Extracts were washed with water (2x) to neutral pH, dried with anhydrous sodium-sulphate and then were evaporated to dry residue in nitrogen stream (Minnikin, 1975).

Identification of methyl-esters of fatty acids by gas chromatography/mass spectrometry

The composition of fatty acids (FA) and (MEFA) was determined by the combined method of gas chromatography/mass spectrometry (GC/MS).

Gas chromatograms were obtained using a Varian 3400 instrument, on a non polar DB-5 column. Carrier gas hydrogen, 1ml/min measured at 210^{o}. Column temperature was linearly programmed from 60^{o} to 285^{o} at 4.3^{o}/min. Detector FID 300^{o}. Mass spectra were recorded on a Finnigan-Math instrument, model 8230, employing both the electron impact (70 eV) and chemical ionization (with *i*-butane) technique.

GC-MS data were analyzed using AMDIS program version 2 and our compilation library of 3833 spectra of some 1666 compounds (mostly terpenes) with included Retention (Kovatch) Indexes. Retention Indexes were calibrated using a series of n-alkanes C_8H_{18}-$C_{24}H_{50}$ under equal chromatographic conditions as used for analysis (Stein, 1999).

RESULTS AND DISCUSSION

Results of suspension test (dilution-neutralization method) showed significantly higher resistance of spores of *Bacillus cereus* in comparison to *Bacillus subtilis*, where concentrations of 0.05% and 0.1% of PAA (0.5% and 1% of "Germiper") after 60 min and 30 min were effective against spores of *Bacillus cereus* while at the same time both concentrations were effective after 15 minutes

(0.05% PAA – 0.5% of "Germiper") and almost immediately – (0.1% PAA- 1% of "Germiper") against spores of *Bacillus subtilis* (Figures 1. and 2.).

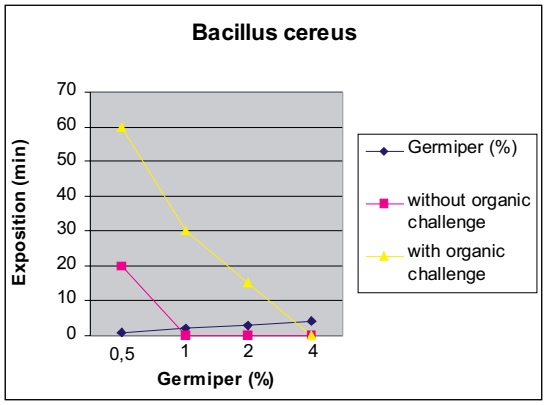

Figure 1. Evaluation of sporicidal effect of PAA on *Bacillus cereus* (initial cell number in the inoculum was $4x10^5$, in both samples)

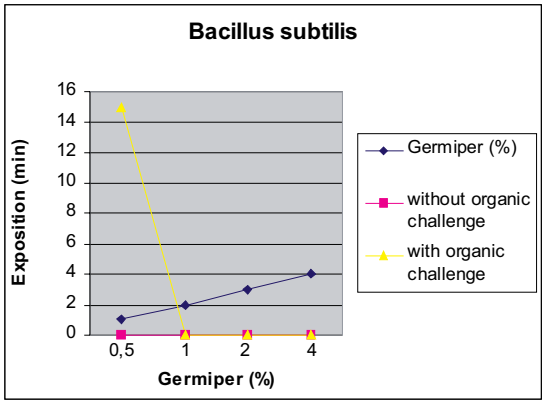

Figure 2. Evaluation of sporicidal effect of PAA on *Bacillus subtilis* (initial cell number in the inoculum was 10^6 in both samples)

In addition, we have also made microphotographs of spores of *Bacillus cereus* and *Bacillus subtilis*, before and after treatment with PAA- *Schöefer-Fulton coloring, original light microscopy, approx. magnification 1200x* (Photos 1. and 2.).

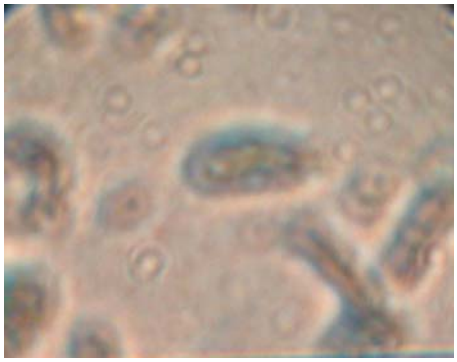

Photo 1. Spores of Bacillus subtilis before treatment, 1200x

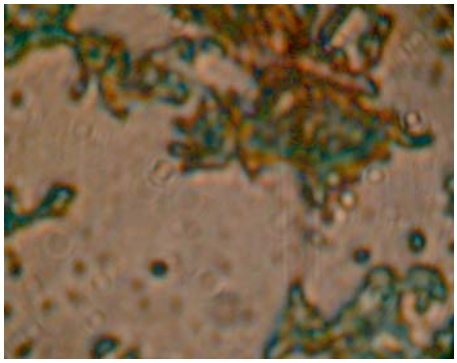

Photo 2. Spores of Bacillus subtilis after treatment, 1200x

In these photos it can be seen that after treatment with PAA, spores of *Bacillus subtilis* are dark in appearance and some aggregation of spores has occurred.

Chemical characterization of disinfectant "Germiper" showed that average content of PAA was 10.14%, while average content of active oxygen was 11.22%. From the obtained results we concluded that spores of *B. cereus* are more resistant to the action of PAA, then spores of *B. subtilis* (Figures 1. and 2.). In the presence of organic challenge (3 g/L of defibrinated horse blood) the sporicidal activity against *B. subtilis* was achieved with a starting concentration of "Germiper" of 0.5% (0.05% PAA) and exposition of only 15 min. This resulted in a log reduction, compared to the initial bacterial number, of 6 log (Figure 2.). Taking into consideration spores of *B. cereus*, it is estimated that complete sporicidal activity was achieved within 30 min with an active concentration of "Germiper" of 1% (0.1% PAA). This achieved log reduction, compared to initial bacterial number of 5 log (Figure 1.). Since EU standards for the evaluation of disinfectants sporicidal activity CEN/TC 216, EN 216003 (Cremieux *et al.,* 2001) define that reduction of bacterial number after performed disinfection should be at least 4 log within 120 minutes, the obtained results confirme a high oxidative power and potency of PAA. The PAA and hydrogen peroxide combination has shown to have a bactericidal and detaching effect on polymicrobial biofilms grown in a continuos culture (Alasri *et al.,* 1992). Finally, we also concluded that spores of *B. cereus* are two times more resistant then spores of *B. subtilis.*

Quantitative test AFNOR (NF T 72-230 and 72-231) also defines the sporicidal effect as a reduction of 5 log in 1 h at 20°C or in 5 minutes at 75°C and for industrial use – 3 log, in 1 h, at 20°C (Cremieux *et al.,* 2001).

Results for the identification of MEFA and detection of quantitative changes in *Bacillus cereus*, by GC/MS are given in Fig. 4 and Fig. 5. The identification of MEFA was performed by Supelco standard of methyl-esters of bacterial fatty acids (Figure 3.).

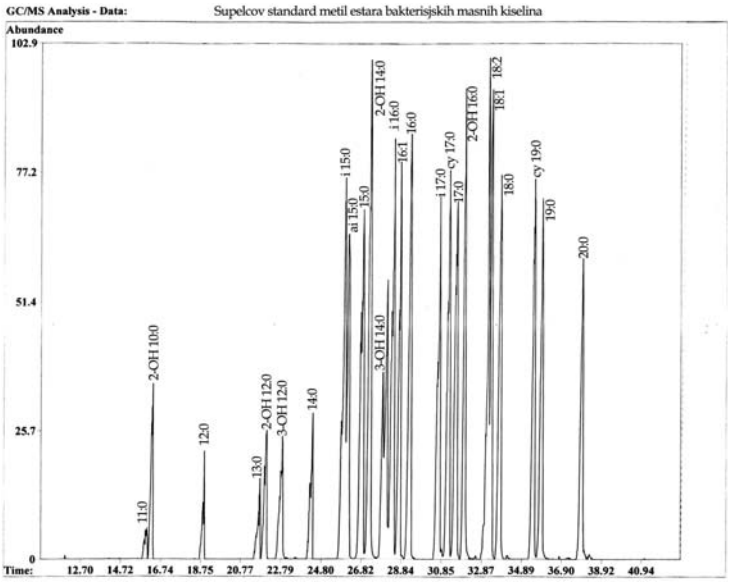

Figure 3. Supelco standard of methyl-esters of bacterial fatty acids

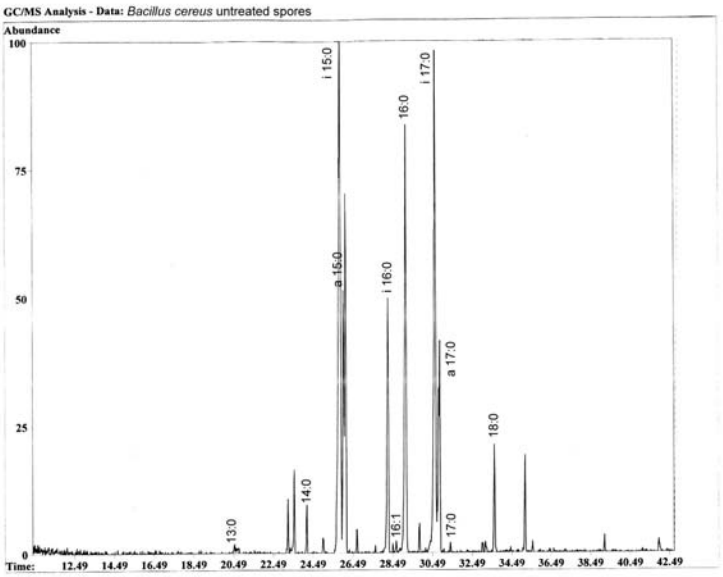

Figure 4. *B. cereus* – untreated spores

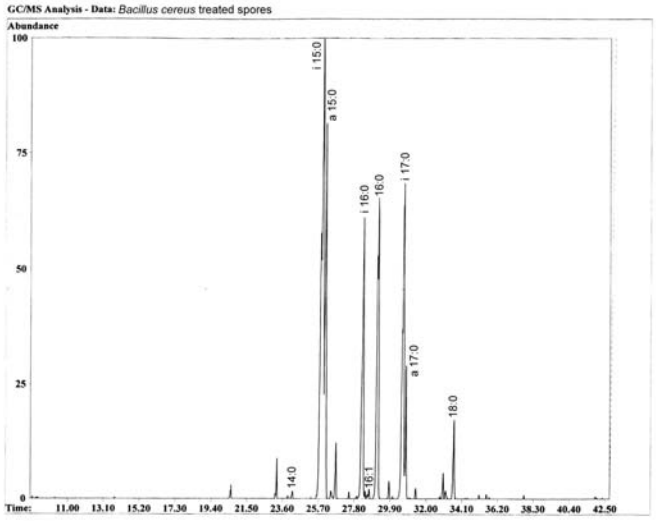

Figure 5. *B. cereus* – spores treated with PAA

Thus, after treatment with a previously confirmed sporicidal concentration of PAA the obtained results showed a decrease of fatty acids 16:0. 16:1, *iso* 17:0 and *anteiso* 17:0. In Figures 6., 7., and 8. In *Bacillus subtilis* culture can be seen that after the treatment with sporicidal concentration of PAA, a significant decrease of fatty acids *anteiso* 15:0 and *anteiso* 17:0 occurred.

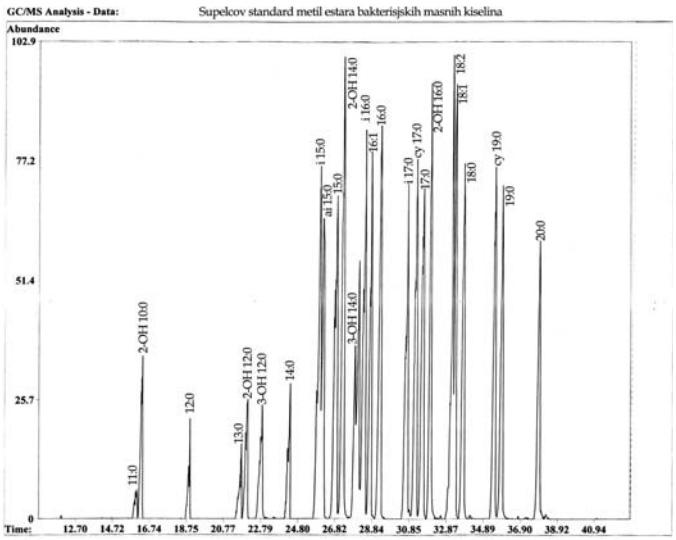

Figure 6. Supelco standard of methyl-esters of bacterial fatty acids

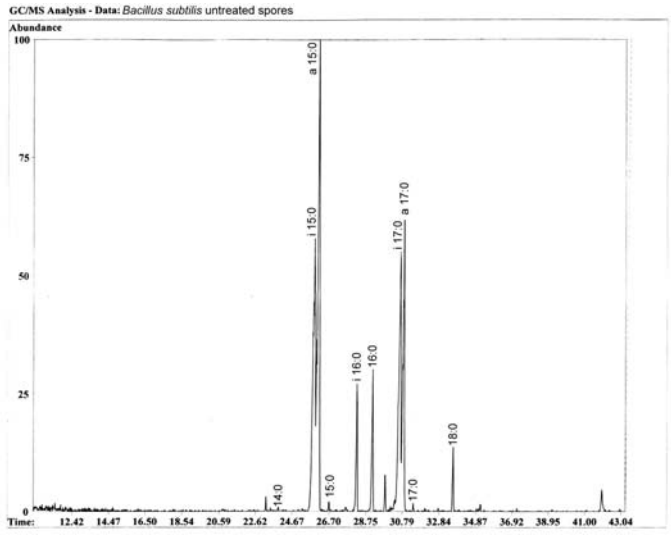

Figure 7. *B. subtilis* – untreated spores

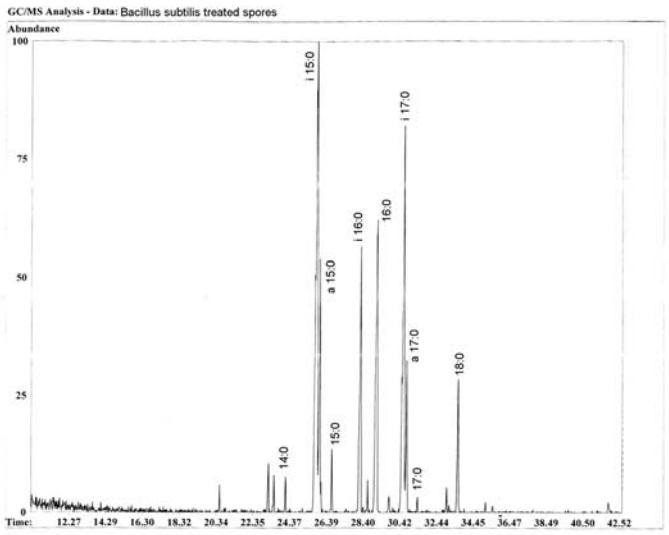

Figure 8. *B. subtilis* – spores treated with PAA

In Figure 9. the changes of percentage of MEFA in *Bacillus cereus* can be seen, which is also presented in Table 1. where quantitative changes of MEFA, before and after treatment with PAA, are shown.

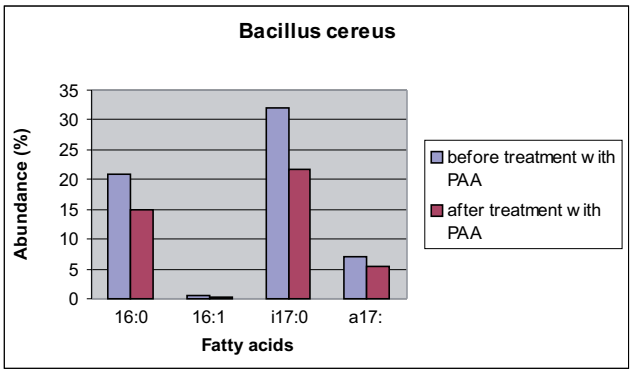

Figure 9. Quantitative changes of fatty acids in *B. cereus*, before and after treatment with PAA

Table 1. Concentration (%) of fatty acids in *Bacillus cereus* before and after treatment with PAA

Fatty acid	Before treatment (%)	After treatment (%)	Decrease (folds)	Decrease (%)
16:0	20.8	15.0	1.6	28.0
16:1	0.64	0.2	3.5	71.5
i17:0	31.9	21.6	1.5	32.4
a17:0	7.0	5.5	1.3	21.3

Consequently, in *B. cereus*, the highest rate of decrease occurred in monounsaturated fatty acid 16:1 (3.5 folds), with a decrease of 71.5%. The changes of percentage of MEFA in *Bacillus subtilis* before and after treatment are presented in Figure 10 and Table 2.

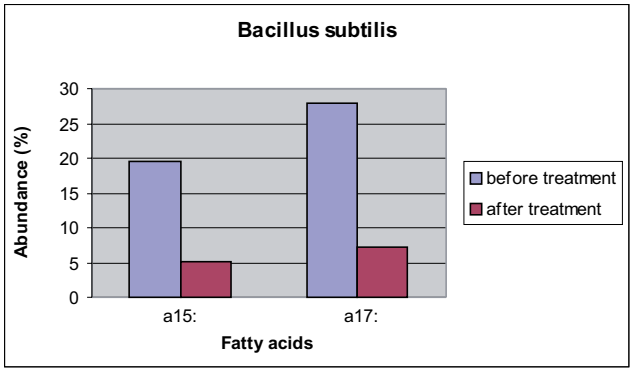

Figure 10. Quantitative changes of fatty acids in *B. subtilis*, before and after treatment with PAA

Table 2. Concentration (%) of fatty acids in *Bacillus subtilis* before and after treatment with PAA

Fatty acid	Before treatment (%)	After treatment (%)	Decrease (folds)	Decrease (%)
a15:0	19.5	5.1	3.8	73.7
a17:0	28.0	7.2	3.5	74.4

In *B. subtilis*, the highest rate of decrease occurred in fatty acid *anteiso* 15:0 (3.8 folds). with a decrease of 73.7%. Further on, in Table 3. the composition of *Bacillus cereus* and *Bacillus subtilis* fatty acids (Ratledge and Wilkinson, 1988) is given.

It is clear that in both bacterial strains *iso*-branched and *anteiso*-branched fatty acids dominate and that those fatty acids are subject to aforementioned quantitative changes after treatment with PAA.

In Figure 11. it can be seen that one of the possible PAA acting mechanisms on fatty acids could be the formation of epoxydes by acting on double bonds (Sinadinovic *et al*., 2001).

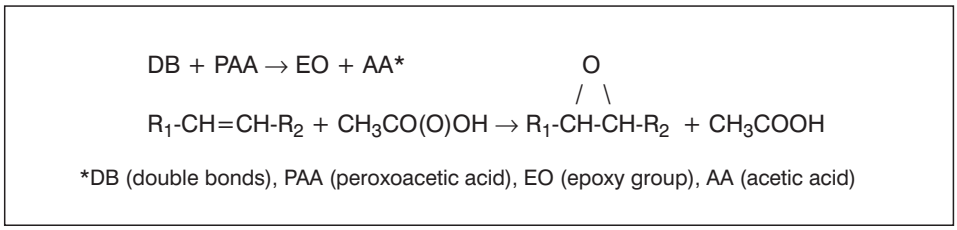

Figure 11. Reaction of PAA with double bonds in fatty acids and formation of epoxy group

Investigations in the field of industrial chemistry (production of polymers) showed that on average 90% of double bonds are converted into epoxy groups (Petrovic *et al.,* 2002). It is also confirmed that PAA reacts with solutes that contain double bonds and can disrupt the chemiosmotic function of the lipoprotein cytoplasmic membrane transport through rupture or dislocation of cell walls, which impedes cellular activity (Baldry and Fraser, 1988).

On the basis of the obtained results we can conclude that the confirmed quantitative changes of fatty acids in bacterial spores of *B. cereus* and *B. subtilis* could have a certain role in the modification of chemodynamics, metabolism and selective permeability of cell membranes, which undoubtly facilitate the penetration of tested biocide (PAA) into the cell interior and provoke further destruction of vital cellular structures. Certainly, further research is needed to highlight specific mechanisms of action of peroxygen disinfectants on the lipid component of bacterial spores.

Table 3. Composition of *B. cereus* and *B. subtilis* fatty acids

Spec.	Straight chain C-even				Straight chain C-odd	Iso-branched				Anteiso-branched			Al. Acids
	14:0	16:0	16:1	others		i14:0	i15:0	i16:0	i17:0	others	a15:0	a17:0	others	
B. cereus %	3	12	5	0	0	10	23	15	7	4	12	7	2	0
B. subtilis %	Trace	5	0	0	0	3	14	10	14	0	40	14	0	0

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ISPITIVANJE DEJSTVA PERSIRĆETNE KISELINE NA LIPIDNU KOMPONENTU BAKTERIJSKIH SPORA I DOPRINOS STANDARDIZACIJI TESTA ZA OCENU EFIKASNOSTI

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SADRŽAJ

Cilj ovog istraživanja je bio da se stekne potpuniji uvid u dejstvo peroksigenih dezinfektanata na bazi peroksosirćetne kiseline (u daljem tekstu: PSK) na lipidnu komponentu sporogenih formi mikroorganizama, za koje je poznato da pokazuju veću otpornost od vegetativnih oblika. *Bacillus cereus ATCC 11778* i *Bacillus subtilis NCTC 10480* su odabrani kao referentni sojevi – bioindikatori, na kojima je ispitivano delovanje PSK (nakon formiranja spora).

Nakon tretmana spora pomenutih bakterijskih sojeva sa PSK, izolovane su masne kiseline iz netretiranih i tretiranih spora i analizirane su promene u lipidnoj komponenti. Dobijeni metil-estri masnih kiselina (MEMK) analizirani su pomoću gasne hromatografije i masene spektrometrije (GC/MS), uz poređenje sa standardom bakterijskih MEMK. Dobijeni rezultati su ukazali da je kod *B. cereus ATCC 11778* nakon tretmana došlo do kvantitativne promene, odnosno smanjenja količine masnih kiselina sa 16 i 17 C atoma, odnosno 16:0, 16:1, *anteiso* 17:0, *iso* 17:0. Kod *B. subtilis NCTC 10480* došlo je do značajnog smanjenja količine masnih kiselina sa 15 i 17 C atoma, odnosno *anteiso* 15:0 i *anteiso* 17:0.