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COMPARATIVE STUDY OF ANTIBACTERIAL ACTIVITY OF PEROXYDISUCCINIC ACID, HYDROGEN PEROXIDE AND THEIR MIXTURE

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Introduction

It is known that reactive oxygen species (ROS) generated *in vivo* by cell aerobic metabolism cause multiple damage in different cell organelles and kill not only obligate anaerobes and microaerophilles, but also aerobes [1, 2]. ROS generated by phagocytes and representatives of normal microflora are an important component of macroorganism defense from most pathogens, which is explained by their ability to damage different biological structures [3].

ROS have high reactivity and let us use them *in vitro* as effective biocides.

Hydrogen peroxide is widely used in many industries, in particular, in medicine and veterinary as antiseptic and disinfectant agent due to its safety for environment and broad spectrum of antimicrobial activity including spore-forming bacteria [4].

However, in the recent years certain decrease of background sensitivity of microorganisms to hydrogen peroxide and occurrence of resistant strains of pathogenic microorganisms to this agent has been noted.

The authors anticipate the existence of two dose-dependent mechanisms of antimicrobial activity of hydrogen peroxide: in the first one, at the influence of hydrogen peroxide's low concentrations, cells' death occurs mainly due to DNA damaging, whereas in the second one, at the influence of high concentrations, cells' death occurs due to other organelles' damaging [1].

The high cytotoxicity level of hydrogen peroxide in low concentration is explained by formation of radical oxygen species, in particular, hydroxyl radicals.

The hydroxyl radical is a power oxidizer with a short lifetime, which reacts with proteins, lipids, and nucleic acids.

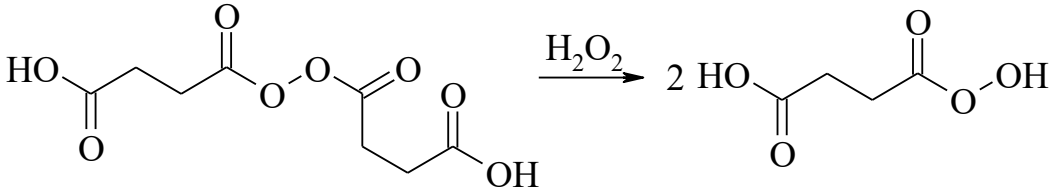
Indeed, in high quantities its damaging effect also involves other cell structures [5].

It is should be noted that the possibility of ROS forming in the cell is limited by low concentration of transition metal ions even with high content of hydrogen peroxide.

Bactericidal effect of hydrogen peroxide is also limited by catalase activity level and existence of intracellular antioxidant pool [6].

In the paper [7] the authors have suggested that oxygen coupled by metal ion in aerobic metabolism can react with decarboxylation of ketoglutaric acid and form monoperoxysuccinic acid (MPSA) that can be a hydroxylating agent [6].

It is known that MPSA is a highly reacting and unstable compound and it can be easy obtained by perhydrolysis reaction (with hydrogen peroxide excess) of commercially available peroxydisuccinic acid (PDSA) [9]:



PDSA is rather stable when stored at normal temperature, it is insensitive to mineral acids, shock and friction, which insures certain safety level when working with this substance.

Thus, the system PDSA - H_2O_2 can be regarded as an original model to obtain hydroxylating agent in solution *in situ* that a priori has enhanced bactericidal properties.

Thus, it is logically to suppose that in high quantities MPSA can cause damage not only DNA, but its oxidizing action will also affect other cell elements.

Moreover, significant increase of bactericidal effect of hydrogen peroxide in case of MPSA presence is possible due to inhibition of bacteria's catalase activity generated in ROS reaction, and opens new possibilities to enhance the efficiency of hydrogen peroxide.

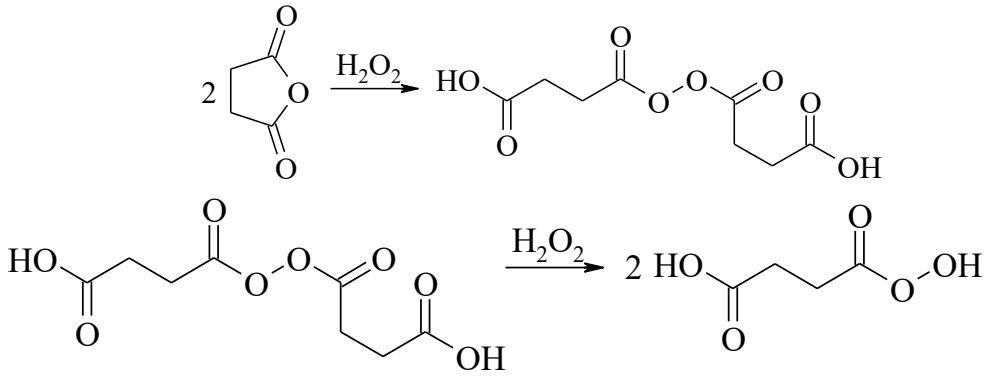
This approach may certainly be aimed at elimination of factors that decrease bactericidal effect of hydrogen peroxide and implicates inhibition of cell antioxidant defense, like in the above-mentioned case with catalase, or formation of optimal condition for ROS generation, in particular, hydroxyl radicals by selection of respective PDSA concentrations.

All the facts mentioned above have determined the aim of this study.

The **aim** of this work is to carry out a comparative study of antimicrobial activity of hydrogen peroxide, peroxydisuccinic acid (PDSA), monoperoxysuccinic acid (MPSA), and mixture of PDSA and hydrogen peroxide (H_2O_2).

Materials and methods

The substances of peroxydisuccinic acid (PDSA) and monoperoxysuccinic acid (MPSA) were prepared by well known methods [8, 9]:



PDSA (bis(β-corboxipropionyl)peroxide, $HOC(O)(CH_2)_2C(O)O\text{-}OC(O)(CH_2)_2C(O)OH$, melt. point of 132-132.5 ºC, act.oxygen 6.8 %; MPSA (monoperoxybutanedioic acid), $HOC(O)(CH_2)_2C(O)OOH$, melt. point of 107 ºC (decomp.); act.oxygen 11.9%, pKa_1=6.21±0.02; pKa_2=8.73±0.015 (n=30, P=0,95) at I=0.02 mol/l, rate constant of spontaneous decomposition in water solution k=9.5·10^{-7} sec^{-1} (pH 4.7, 293K) [10].

The following test-strains were used to assess antimicrobial activity of the agents: *Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Pseudomonas aeruginosa ATCC 9027, Basillus cereus ATCC 10702, Basillus cereus ATCC 96, Basillus subtilis ATCC 6633, Proteus vulgaris ATCC 4636, Candida albicans ATCC 885/653,* and *Candida albicans ATCC 10231.*

All disinfectant agents were diluted in distilled water at 40 ºC and stirred. The microbial burden was 2·10^9 CFU/ml of the medium, and for kinetic studies 10^5 CFU/ml of the medium, it was standardizing according to McFarland standard.

Microorganisms' resistance to disinfectant agents was determined by seeding of the diluted cell suspension on agar medium after their incubation with MPSA, PDSA, H_2O_2, and also after MPSA generation in the reaction between PDSA and H_2O_2, and seeding of the cell suspension with exclusion of these factors was taken as control.

18-24-hour microorganism culture was taken into work. Mueller-Hinton agar and MPA were used for bacteria. Sabouraud agar was used for *Candida albicans*.

Agar well diffusion method was applied. Determination of antimicrobial activity of the agents were carried out on two layers of solid medium in Petri dishes.

"Starvation" non-seed media (agar-agar, water, salts) were used for lower layer.

The volume of the lower layer was 10 ml, on the surface of which 3 thin-wall cylinders from stainless steel with diameter and height of 10 mm were installed strictly horizontally.

The upper-layer medium filled up the volume around the cylinders and consisted of culture agar-medium melted and cooled to 40 ºC, where respective standard of twenty-four hour test-strain culture was seeded.

Preliminary, the upper-layer has mixed well to form a homogeneous mass.

Cylinders were taken out with sterile tweezers after solidification of the medium and test solution was added into the wells formed taking into consideration its volume (0.275±0.025 ml).

The upper-layer volume was 15 ml. Petri dishes were dried for 30-40 min at room temperature and transferred into the thermostat for 18-24 h.

In kinetic study of microorganism's death rate due to incubation period with peroxide solution tested, the bactericidal action was stopped with 5 % sodium thiosulfate sterile solution. All studies were carried out in 3 replicates. In statistical analysis methods, significance level P was equal to 0.95.

Student's test was used for statistical processing of the data obtained.

Results and discussion

Results of the studies carried out in order to determine antimicrobial activity of test peroxide solutions and their compositions are represented in Tables 1-2.

Table 1 - Dependence between diameters of microorganism growth inhibition zones and concentration of PDSA and MPSA, mm

Concentration of the agent, % wt.	*S. aureus* 25923	*E. coli* 25922	*P. aeruginosa* 27853	*B. cereus* 96	*Candida albicans* 10231
PDSA					
1.0	24±2.3	24±2.2	19±1.8	26±2.5	19±1.7
0.5	24±2.4	24±2.1	18±1.7	25±2.2	18±1.8

0.25	23±2.1	21±2.0	17±1.5	25±2.3	18±1.7
0.1	22±2.0	20±1.8	16±1.4	24±2.2	17±1.6
0.05	20±1.9	19±1.9	16±1.6	24±2.4	17±1.5
MPSA					
0.25	22±2.0	20±2.0	17±1.5	25±2.4	18±1.8
0.1	22±1.9	20±1.8	18±1.7	24±2.2	16±1.6
0.05	20±1.8	19±1.9	16±1.6	23±2.3	16±1.5
0.01	19±1.7	17±1.6	16±1.5	24±2.4	15±1.5
0.005	18±1.8	16±1.5	15±1.5	22±2.0	13±1.4

Table 2 - Dependence between diameters of microorganism growth inhibition zones and concentration of peroxide, mm

Concentration of the agent, % wt.	*S.aureus* 25923	*E.coli* 25922	*P.aeruginosa* 9027	*B.cereus* 10702	*B.subtilis* 6633	*C.albicans* 885-663
H_2O_2 3%	12±1.5	13±1.4	-	15±1.5	15±1.4	-
H_2O_2 6%	12±1.4	13±1.4	-	14±1.4	14±1.5	-
PDSA 1%	23±2.1	25±2.4	20±2.0	30±2.8	35±3.4	15±1.4
H_2O_2 3% + 1% PDSA	30±2.8	28±2.5	25±2.2	30±2.9	35±3.5	17±1.5
H_2O_2 6% + 1% PDSA	35±3.0	30±2.9	25±2.4	32±3.1	35±3.2	20±1.8
H_2O_2 1,5% + 0,5% PDSA (2-fiold dilution)	25±2.4	30±2.7	24±2.4	33±3.2	34±3.1	15±1.4
H_2O_2 0,6% + 0,2% PDSA (5-fiold dilution)	25±2.5	27±2.6	23±2.2	33±3.1	35±3.2	17±1.6
H_2O_2 3% + 0,5% PDSA	24±2.1	28±2.5	24±2.4	29±2.8	34±3.3	15±1.5

" - " – no microorganism growth was observed

As it can be seen from Tables 1-2, antimicrobial activity of PDSA and MPSA is practically equal and exceeds significantly that one of H_2O_2.

Use of PDSA and H_2O_2 mixture in the experiment led to increase of microorganisms' sensitivity to hydrogen peroxide due to MPSA generation and, probably, ROS generated in cross-reaction between MPSA and excess of hydrogen peroxide.

This hypothesis is particularly confirmed by the fact that dilution of the reaction mixture PDSA + H_2O_2 led to significant increase of bactericidal effect of the system tested, i.e. synergistic effect was observed.

For the evaluation of the dependence between microorganisms' death rate and the period of their incubation with ROS, we used the following test-strains: *S. aureus ATCC 25923, E. coli ATCC 25922, P. aeruginosa ATCC 9027, B. cereus ATCC 10702, B. cereus ATCC 10702,* and *C. albicans ATCC 885/653* (Table 3).

It has been found that treatment of *S. aureus ATCC 25923, E. coli ATCC 25922* test-strains for 60 min with 3% (and even 6% H_2O_2 for *E. coli*) does not cause total death of microorganisms.

The fact of resistance of spore-forming cultures *B. cereus, P. aeruginosa* and *C. albicans* to hydrogen peroxide should be noted, in 60 min of their incubation with 6% H_2O_2, the number of viable cells decreased from

5.0 to 1.58 lg CFU/ml, as well as to 3.00 (to 1.84 for 120-min exposition) and 1.65 lg CFU/ml, respectively.

However, pretreatment of test-stains by 5-fold diluted mixture of 3% H_2O_2 and 1% PDSA for 60 min caused total death of *S. aureus* and *E. coli*, and decreased the number of viable cells of *B. cereus* spore-forming cells and relatively resistant to hydrogen peroxide cultures of *P. aeruginosa* и *C. albicans* from 5.00 to 1.34 and 0.95 lg CFU/ml, respectively, and in 120 min to their total death (Fig. 1-3).

Table 3 - Survival of *S.aureus* 25923 and *E.coli* 25922 depending on the time of bacterial cell incubation in presence of H_2O_2, PDSA, as well as in H_2O_2+PDSA system. Seeding dose of microorganisms was 10^5 CFU/ml of the medium.

Concentration of the agent, % wt.	*S.aureus* 25923					*E.coli* 25922				
	5'	10'	30'	60'	120'	5'	10'	30'	60'	120'
H_2O_2 3%	10^2±50	10^2±50	10^2±50	10^2±50	-	10^4±10^1	10^4±10^1	10^3±10^1	10^2±50	-
H_2O_2 6%	10^3±10^1	10^2±50	10^2±50	-	-	10^3±10^1	10^3±10^1	10^2±50	10^2±50	-
PDSA 1%	27±15	25±14	6±4	-	-	48±25	24±15	4±3	-	-
H_2O_2 3% + 1% PDSA	34±18	12±6	2±2	-	-	34±20	16±15	-	-	-
H_2O_2 6% + 1% PDSA	22±15	10±5	-	-	-	29±30	12±7	-	-	-
H_2O_2 1,5% + 0,5% PDSA	14±7	12±7	6±3	-	-	29±15	21±10	20±10	-	-
H_2O_2 0,6% + 0,2% PDSA	26±15	11±6	3±2	-	-	34±18	22±11	14±8	-	-
H_2O_2 3% + 0,5% PDSA	21±10	14±8	5±3	-	-	27±15	17±9	10±6	-	-

" - " – no microorganism growth was observed

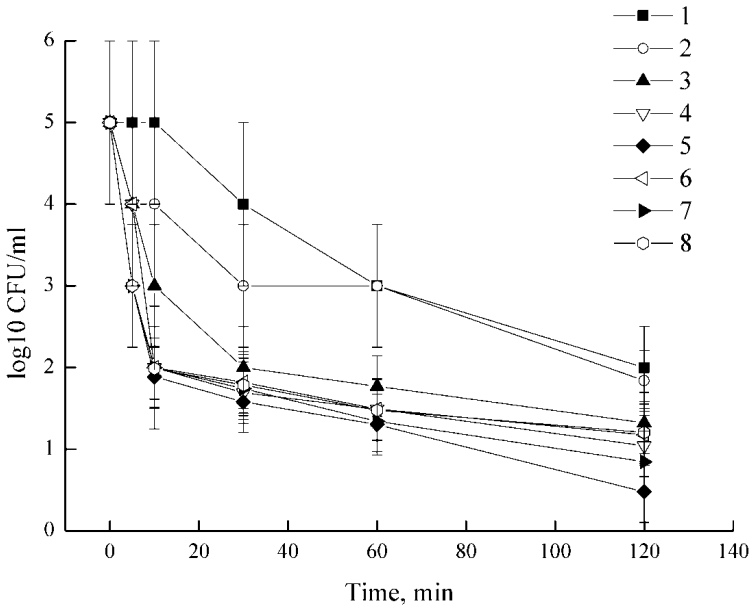


Fig. 1. Survival of *P. aeruginosa ATCC* 9027 (*lg CFU*/ml) depending on incubation time (min) in H_2O_2, PDSA and PDSA+H_2O_2 systems

1 - H_2O_2 3%, 2 - H_2O_2 6%, 3 - PDSA 1%, 4 - H_2O_2 3% + 1% PDSA, 5 - H_2O_2 6% + 1% PDSA, 6 - H_2O_2 1.5% + 0.5% PDSA (2-fold dilution), 7 - H_2O_2 0.6% + 0.2% PDSA (5-fold dilution), 8 - H_2O_2 3% + 0.5% PDSA

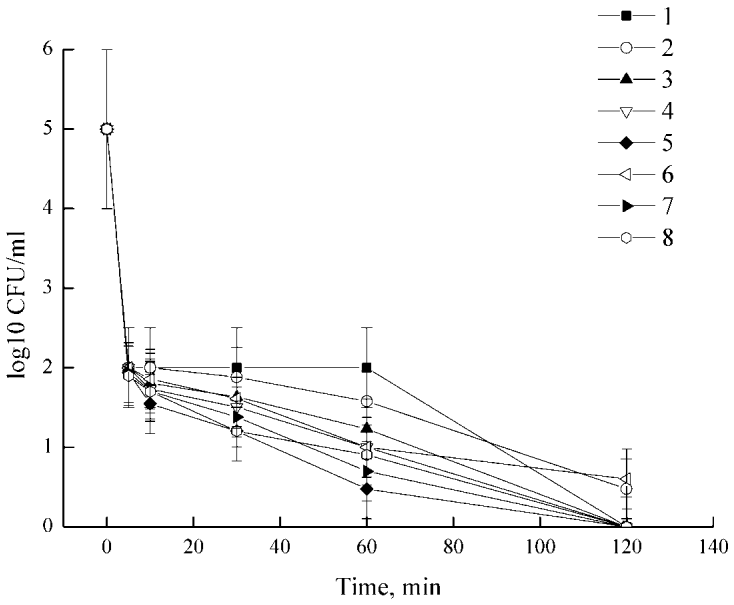


Fig. 2. Survival of *Bacillus cereus ATCC 10702* (*lg CFU*/ml) depending on incubation time (min) in H_2O_2, PDSA and PDSA+H_2O_2 systems

1 - H_2O_2 3%, 2 - H_2O_2 6%, 3 - PDSA 1%, 4 - H_2O_2 3% + 1% PDSA, 5 - H_2O_2 6% + 1% PDSA, 6 - H_2O_2 1.5% + 0.5% PDSA (2-fold dilution), 7 - H_2O_2 0.6% + 0.2% PDSA (5-fold dilution), 8 - H_2O_2 3% + 0.5% PDSA

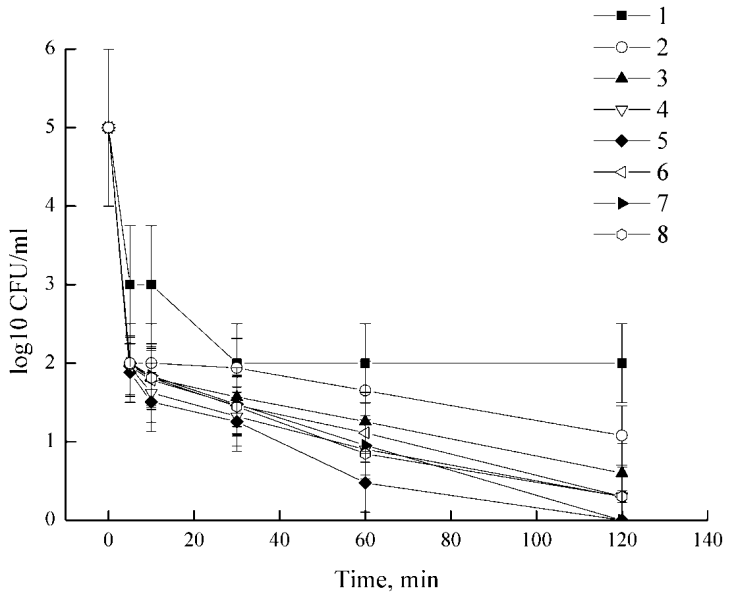


Fig. 3. Survival of *Candida albicans* (*lg CFU*/ml) depending on incubation time (min) in H_2O_2, PDSA and PDSA+H_2O_2 systems

1 - H_2O_2 3%, 2 - H_2O_2 6%, 3 - PDSA 1%, 4 - H_2O_2 3% + 1% PDSA, 5 - H_2O_2 6% + 1% PDSA, 6 - H_2O_2 1.5% + 0.5% PDSA (2-fold dilution), 7 - H_2O_2 0.6% + 0.2% PDSA (5-fold dilution), 8 - H_2O_2 3% + 0.5% PDSA

Discussion. Materials of the studies conducted demonstrate in a conclusive way that biocidal effect of synergetic mixtures of PDSA and hydrogen peroxide is higher manifold and more quickly achievable than with hydrogen peroxide only.

Significant increase in bacteria death rate can be explained by high reactivity of ROS generated in cross-reactions between MPSA (formed in the result of PDSA peroxihydrolysis) and excess of hydrogen peroxide in the solution, and in spite of their short lifetime, they cause fast and nonspecific damage of cell structures.

One of the factors that limit ROS formation and, as a consequence, its biocidal action, is lack of free transition metal ions.

Conclusions. Results of this study demonstrate the possibility to overcome this obstacle by creation of optimal conditions for ROS generation with another peroxide compound of PDSA in much lower concentrations.

Apparently, the combination of a short lifetime and fast biocide activity of ROS is of special interest, as it allows us to consider radical-generating systems as a means for development of a highly efficient disinfectant with a broad spectrum of antimicrobial activity and without a tendency to accumulate in the environment.

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Introduction. It is known that reactive oxygen species (ROS) generated *in vivo* by cell aerobic metabolism cause multiple damage in different cell organelles and kill not only obligate anaerobes and microaerophilles, but also aerobes. ROS generated by phagocytes and representatives of normal microflora are an important component of macroorganism defense from most pathogens, which is explained by their ability to damage different biological structures. ROS have high reactivity and let us use them *in vitro* as effective biocides. Hydrogen peroxide is widely used in many industries, in particular, in medicine and veterinary as antiseptic and disinfectant agent due to its safety for environment and broad spectrum of antimicrobial activity including spore-forming bacteria. However, in the recent years certain decrease of background sensitivity of microorganisms to hydrogen peroxide and occurrence of resistant strains of pathogenic microorganisms to this agent has been noted. The **aim** of this work is to carry out a comparative study of antimicrobial activity of hydrogen peroxide, peroxydisuccinic acid (PDSA), monoperoxysuccinic acid (MPSA), and mixture of PDSA and hydrogen peroxide (H_2O_2). **Materials and methods.** The substances of peroxydisuccinic acid (PDSA) and monoperoxysuccinic acid (MPSA) were prepared by well known methods. The following test-strains were used to assess antimicrobial activity of the agents: *Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Pseudomonas aeruginosa ATCC 9027, Basillus cereus ATCC 10702, Basillus cereus ATCC 96, Basillus subtilis ATCC 6633, Proteus vulgaris ATCC 4636, Candida albicans ATCC 885/653,* and *Candida albicans ATCC 10231.* All disinfectant agents were diluted in distilled water at 40 ºC and stirred. The microbial burden was 2·109 CFU/ml of the medium, and for kinetic studies 105 CFU/ml of the medium, it was standardizing according to McFarland standard. Microorganisms' resistance to disinfectant agents was determined by seeding of the diluted cell suspension on agar medium after their incubation with MPSA, PDSA, H_2O_2, and also after MPSA generation in the reaction between PDSA and H_2O_2, and seeding of the cell suspension with exclusion of these factors was taken as control. 18-24-hour microorganism culture was taken into work. Mueller-Hinton agar and MPA were used for bacteria. Sabouraud agar was used for *Candida albicans*. Agar well diffusion method was applied. Determination of antimicrobial activity of the agents were carried out on two layers of solid medium in Petri dishes. In kinetic study of microorganism's death rate due to incubation period with peroxide solution tested, the bactericidal action was stopped with 5 % sodium thiosulfate sterile solution. All studies were carried out in 3 replicates. In statistical analysis methods, significance level *P* was equal to 0.95. Student's test was used for statistical processing of the data obtained. **Results and discussion.**

It has been found that treatment of *S. aureus ATCC 25923, E. coli ATCC 25922* test-strains for 60 min with 3% (and even 6% H_2O_2 for *E. coli*) does not cause total death of microorganisms. The fact of resistance of spore-forming cultures *B. cereus, P. aeruginosa* and *C. albicans* to hydrogen peroxide should be noted, in 60 min of their incubation with 6% H_2O_2, the number of viable cells decreased from 5.0 to 1.58 lg CFU/ml, as well as to 3.00 (to 1.84 for 120-min exposition) and 1.65 lg CFU/ml, respectively. However, pretreatment of test-stains by 5-fold diluted mixture of 3% H_2O_2 and 1% PDSA for 60 min caused total death of *S. aureus* and *E. coli*, and decreased the number of viable cells of *B. cereus* spore-forming cells and relatively resistant to hydrogen peroxide cultures of *P. aeruginosa* и *C. albicans* from 5.00 to 1.34 and 0.95 lg CFU/ml, respectively, and in 120 min to their total death **Conclusions.** The study have determined that antimicrobial effect of reactive oxygen species (ROS) produced by interaction of hydrogen peroxide and peroxydisuccinic acid (PDSA) mixture in the synergistic system is many times higher and faster achievable than hydrogen peroxide or peroxydisuccinic acid only. We have determined the principles of optimum conditions for ROS generation by choice of respective concentrations of peroxydisuccinic acid and hydrogen peroxide. ROS demonstrate high biocidal activity and short lifetime, which allows us to consider the combination of hydrogen peroxide and peroxydisuccinic acid as a promising means for development of a highly efficient disinfectant with a broad spectrum of antimicrobial activity and without a tendency to accumulate in the environment.

Key words: reactive oxygen species (ROS), bacteria, biocide, disinfectant, hydrogen peroxide, peroxydisuccinic acid, synergetic composition.