

Original Research Article

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Antibacterial Activity of Electrochemically Activated Water Solutions on *Pseudomonas aeruginosa* after Four Weeks Storage

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ABSTRACT

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Studies have been carried out to determine the effect of activated aqueous solutions (anolytes and catholytes) on multi-resistant strain of *Pseudomonas aeruginosa* *in vitro* after storage at room temperature. The solutions were obtained by electrochemical activation of water with 0.8% NaCl, and with a combination of 0.4% NaCl and 0.4% Na₂CO₃. The disinfectant Virkon S was used as a control. Cultures were made on Cetrimide agar after different intervals of action of the solutions on *P. aeruginosa* suspensions (10⁶ cells/ml). Anolytes and catholytes tested exhibited high antimicrobial activity. Upon potentiation of the anolytes by addition of 96% ethanol with a final concentration of 1%, bactericidal activity was observed within 2 minutes. The use of catholyte of NaCl to dissolve Virkon S resulted in an increase in its effect compared to its use as an aqueous solution. After storage for 29 days, the solutions tested preserved their antibacterial properties to the maximum extent.

Introduction

The increasing prevalence of strains of pathogenic bacteria showing multiple resistance to antibiotics and also the rapid development of resistance to commonly used disinfectants are serious problems on a global scale. On the other hand, the pollution of the environment with chemical agents used to combat microorganisms in all spheres of human activity is growing. This leads to disturbance of the ecological balance and biodiversity in the nature. In recent years, the electrochemically activated aqueous solutions (EAASs) have been reported to be broad-spectrum and environmentally safe biocidal

products. The scope of their action includes not only bacteria but also spores, viruses and fungi. Gurgulova *et al.*, (2010, 2011), Popova *et al.*, (2016a, 2016c) and others found significant antibacterial activity of such solutions against Gram-positive and Gram-negative bacteria. Atanasov *et al.*, (2014), Karadzhev *et al.*, (2014) and other authors reported for antiviral activity of EAASs, and Tasheva *et al.*, (2010) - for their antimycotic action. Antimicrobial effect of such solutions was found even in environments with high protein content such as biosludge (Dimitrova *et al.*, 2013) and water from lagoons for storing bovine manure (Peev, 2017). The Gram-negative bacteria exhibit higher

resistance to chemical factors than Gram-positive ones primarily due to the protective properties of their outer lipoprotein envelope, as well as accelerated efflux of the toxic compounds. Among them, *Pseudomonas aeruginosa* is distinguished by particularly rapid adaptation to chemical factors and disinfectant solutions, as not only it does not perish in them but in many cases even multiplies. It is a causative agent of difficult to treat infections in humans and animals, as well as nosocomial infections. The increasingly common combination of antimicrobial multi-resistance and enhanced virulence in this and the others causative agents of communicable diseases requires creativity in the development of prophylactic and therapeutic options (Alibert-Franco *et al.*, 2009; Popova, 2016). EAASs can be a reliable perspective in this aspect. Recently, Popova *et al.*, (2016a, 2016b) found high activity of anolyte of NaCl against *P. aeruginosa*.

Because the increasing isolation of multi-resistant to antimicrobials strains of *P. aeruginosa*, the aim of this study was to investigate the possibilities of potentiating the action of electrochemically activated aqueous solutions by adding ethyl alcohol to increase their antimicrobial activity to this microorganism *in vitro*, as well as their effect after four weeks storage at room temperature.

Materials and Methods

Anolytes

Activation time - 12 min: • Anolyte prepared with 0.8% NaCl. • Anolyte prepared with a combined solution of 0.4% NaCl and 0.4% Na₂CO₃. • Anolyte of 0.8% NaCl potentiated by adding 96% ethanol to a final concentration of 1%. • Anolyte of a combination of 0.4% NaCl and 0.4% Na₂CO₃ potentiated by addition of 96% ethanol at a final concentration of 1%.

Catholytes

Activation time - 12 min: • Catholyte prepared with 0.8% NaCl. • Catholyte prepared with a combined solution of 0.4% NaCl and 0.4% Na₂CO₃.

Control

The broad-spectrum disinfectant Virkon S, applied at a final concentration of 0.5%, was used as a positive control.

Microorganisms

A virulent strain of *P. aeruginosa*, isolated from a horse with clinical signs of acute sinusitis, was used. The isolation and identification were conducted in accordance with the international identifier of Bergey (Holt *et al.*, 1994).

Determination of the sensitivity of isolated bacteria to antimicrobial means

It was done through the classic agar-gel diffusion method of Bauer *et al.*, (1966) with standard antibiotic discs (BULBIO - NCIPD Ltd. - Sofia, Bulgaria).

The results were interpreted in the three-tier system of Bauer *et al.*, (1966) after measuring the diameters of inhibitory zones in mm.

Nutrient media

Media from BULBIO - NCIPD Ltd. - Sofia, Bulgaria were used: Mueller Hinton agar for preparation of 24-hour cultures of the bacterial strain and for determination of the sensitivity of isolated bacteria to antimicrobial means, Mueller Hinton broth for liquid cultures, as well as *P. aeruginosa* selective Ceftrimide agar for isolation of the strain and determination of the antimicrobial activity of the tested solutions against this bacterial species.

Determination of the antibacterial effect of the electrochemically activated aqueous solutions

Experiment 1. One ml of *P. aeruginosa* broth culture and 2 ml of 3-fold concentrated nutrient broth were added to 3 ml of each EAAS (anolyte or catholyte). The achieved final concentration of EAAS was 50%. Homogenization and culturing for 24-48 hours at 37°C followed.

Experiment 2. A suspension of *P. aeruginosa* at a concentration of 10^7 cells/ml in an amount of 1 ml was added to 9 ml of each EAAS (undiluted) tested, resulting in a final concentration of 10^6 cells/ml. After various time intervals of action of EAAS (2 min, 5 min, 10 min and 15 min), cultures of each of the samples were made on Cetrinide agar and incubated at 37°C for 24-48 h under aerobic conditions.

The following controls were set in both experiments. ▪ With application of the same concentration of the tested bacterial strain: sterile distilled water; distilled water with 1% 96° ethanol; distilled water with 0.5% Virkon S; catholyte with 0.5% Virkon S; catholyte without Virkon S; Mueller Hinton broth; Cetrinide agar. ▪ Without microorganisms: the same variants, as well as 100% anolyte and 100% catholyte.

The EAASs used were stored for 29 days at room temperature in the dark and the experiments were repeated to assess to what extent they retained their activity.

Each experiment was performed in triplicate.

Results and Discussion

The physical indicators pH, oxidation - reduction potential (ORP) and temperature of the EAASs tested are presented in Table 1.

As can be seen from the table, the pH value was lowest in the anolyte of NaCl (2.47), and the highest - in the catholyte of NaCl and Na₂CO₃ (11.95), very similar to this of catholyte with NaCl (11.91). The highest ORP (1000 mV) was measured in the anolyte obtained with 0.8% NaCl, followed by that with a combination of NaCl and Na₂CO₃ (439 mV). In the catholytes, the lowest ORP was registered in that obtained with 0.8% NaCl (-368 mV), followed by the catholyte with a combination of NaCl and Na₂CO₃ (-323 mV). After 29 days of storage at room temperature, the change in pH of the solutions was negligible.

However, alterations were observed in values of their ORP, which were significantly lowered than those measured immediately after their preparation.

The results, presented in Table 2, show that the strain of *P. aeruginosa* was multi-resistant to antibiotics and sensitive only to certain aminoglycosides and quinolones.

The results of the investigations for antibacterial activity of EAASs against *P. aeruginosa* in broth and on Cetrinide agar are shown in Table 3 and some of them - in Figures 1 and 2. After storage of EAASs at room temperature for 29 days, these completely retain their antimicrobial activity. The data fully coincide with those established immediately after their preparation. It can be seen from the table that EAASs tested showed high antimicrobial activity even after 4 weeks of storage, which was the same as that on the day of their production.

From the data presented, it can be seen that the anolyte prepared with 0.8% NaCl, as well as that with 0.4% NaCl and 0.4% Na₂CO₃ applied at a final concentration of 50% and 100%, have a bactericidal effect on the multi-resistant strain of *P. aeruginosa*.

Table.1 Physical indicators of the anolytes and catholytes used

Starting composition		pH	ORP, mV	t°C
Aqueous sodium chloride solution 0.8%	before electrolysis	8,92	218	22,1
	anolyte - first day	2,47	1000	20,7
	after 29 days	2,52	263	21,5
	catholyte - first day	11,91	-368	20,7
	after 29 days	11,42	-146	20,7
Aqueous solution of sodium chloride (0.4%) and sodium carbonate (0.4%)	before electrolysis	11,33	109	18,5
	anolyte - first day	10,74	439	20,6
	after 29 days	10,32	193	21,7
	catholyte - first day	11,95	-323	19,7
	after 29 days	11,02	-238	21,3

ORP - oxidation-reduction potential

Table.2 Sensitivity of the isolated *P. aeruginosa* to antibacterial means *in vitro*

Antimicrobial mean	Disc contents (µg)	Sensitivity of the strain
Thiamphenicol	30	R
Tetracycline	30	R
Lincomycin	15	R
Penicillin	15	R
Oxacillin	1	R
Amoxicillin+Clavulanic acid	10	R
Ampicillin	10	R
Cefuroxime	30	R
Cefotaxime	30	R
Kanamycin	5	R
Novobiocin	30	R
Gentamicin	10	S
Amikacin	10	S
Enrofloxacin	5	S
Ciprofloxacin	5	S
Sulfamethoxazole+Trimethoprim	23,75/1,25	I

S - sensitive; I - intermediate; R – resistant

Table.3 Antibacterial action of EAASs stored for 29 days - growth of *P. aeruginosa* in broth after exposure to 50 % EAASs, and on Cetrinide agar after various intervals of exposure to undiluted EAASs

Sample.№	Type of activated solution	Growth in broth	Growth on Cetrinide agar			
			Time of impact - min			
			2	5	10	15
1	anolyte of NaCl	-	-	-	-	-
2	anolyte of Na ₂ CO ₃	-	-	-	-	-
3	1% 96° ethanol in anolyte of NaCl	-	-	-	-	-
4	1% 96° ethanol in anolyte of Na ₂ CO ₃	-	-	-	-	-
5	1% 96° ethanol in distilled water	+	+	+	+	+
6	0,5% Virkon S in catholyte of NaCl	-	-	-	-	-
7	0,5% Virkon S in distilled water	+	-	-	-	-
8	catholyte of NaCl	+	+	+	+	-
9	catholyte of Na ₂ CO ₃	-	-	-	-	-
K	untreated control	+	+	+	+	+

EAASs - electrochemically activated aqueous solutions

Fig.1 Growth of *P. aeruginosa* on Cetrimide agar after treatment with undiluted EAASs. From left to right above: 1% 96° ethanol in anolyte of NaCl (3 - 1, 2); 1% 96° ethanol in distilled water (5-2, 3); below: 0,5% Virkon S in catholyte of NaCl (6-3); catholyte of NaCl (8 - 1, 2); untreated control (k)

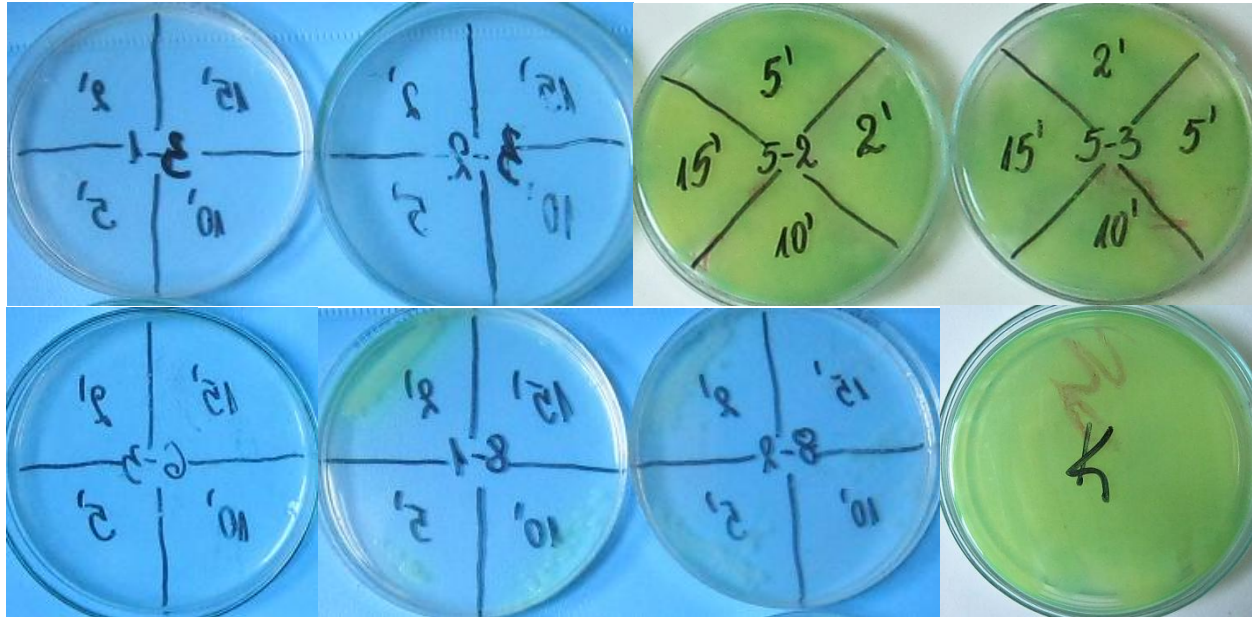
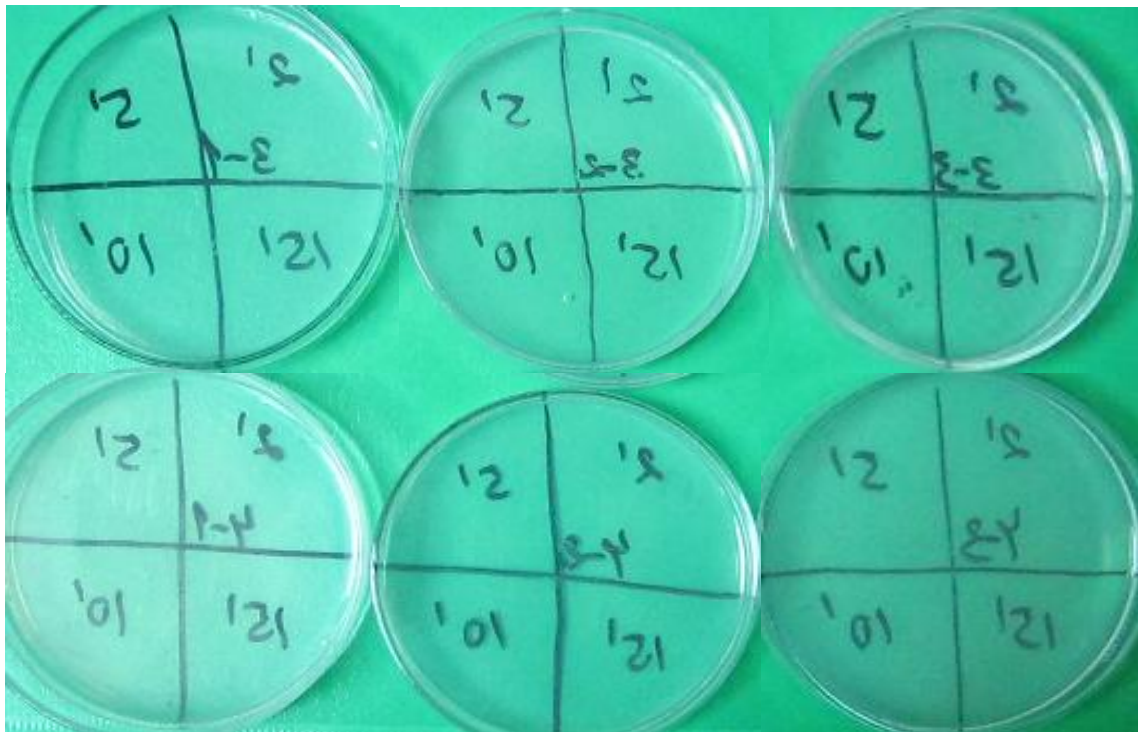


Fig.2 Growth of *P. aeruginosa* on Cetrimide agar after treatment with undiluted EAASs, stored for 29 days. Above: 1% 96° ethanol in anolyte of NaCl (3-1, 2 and 3); below: 1% 96° ethanol in anolyte of Na₂CO₃ (4-1, 2 and 3)



The same anolytes potentiated by the addition of 96% ethanol at a final concentration of 1% also have a rapid bactericidal action. Similar results were also found in samples with catholyte of Na₂CO₃. Under the influence of these five EAASs, *P. aeruginosa* died within 2 minutes as well as after administration of 0.5% Virkon S in catholyte with NaCl. When using 0.5 % Virkon S, dissolved in distilled water, however, growth was observed in some of the broth cultures of *P. aeruginosa* although colonies were not detected on the selective medium even after 48 hours of cultivation.

Our research categorically show that anolytes and catholytes activated with 0.8% NaCl, as well as with a combined application of NaCl and Na₂CO₃, can be used as antiseptics and disinfectants with great success even after storage at room temperature during a relatively long period of four weeks. This disinfection is fast, efficient and safe. In a comparative study of the disinfecting effect of anolytes with a starting composition of sodium chloride alone and a combination of sodium carbonate and sodium chloride, and some of the most commonly used antimicrobials, Gurgulova *et al.*, (2011) indicated that the anolyte has biocidal properties similar to those of peracetic acid, and its high antimicrobial activity is combined with absolutely harmlessness to animals, humans and the environment. The activated substances with biocidal properties and with antimicrobial action against a wide range of microorganisms are formed during electrolysis in the process of preparation of EAASs. The substances resulting from the electrochemical treatment contained in them are in a metastable state for a time interval different for the anolyte and the catholyte, after which they revert to an inactive electrolyte state of the water. Because of their low oxidant content, the anolytes have very little chemical buffering, hence their

environmental safety, both in their production and application, and after their intended use (Atanasov *et al.*, 2014; Ignatov *et al.*, 2015). Upon contact with the microbial cell, the EAASs cause its death mainly through the breakdown of the cell wall integrity, leakage of the intracellular components, violation of the ribosomal apparatus, coagulation of the cytoplasm, and others disabilities (Ashbakh, 2008; Bakhir, 2009a, 2009b).

According to the current studies, the antimicrobial activity of these EAASs can be potentiated by adding 96% ethanol in a final concentration of 1%. The use of catholyte of NaCl to dissolve Virkon S is an opportunity for increasing its effect compared to its use as an aqueous solution. Data from our present studies show also that despite the change in the ORP of the tested solutions, which is essential for their antimicrobial activity, they retain this activity throughout the all studied period of 29 days. This is consistent with the results of our previous studies demonstrating that the antimicrobial activity of anolyte of 3% NaCl is preserved even after storage for weeks at room temperature (Popova *et al.*, 2016b). Obviously, except the ORP, a significant role for the antimicrobial activity of the solutions we investigated has and their hydrogen ion concentration, which was changed very little during the four week study period. Even at a concentration of 50%, the anolytes and the catholyte of Na₂CO₃ are a sure means for safely decontaminating materials containing a virulent multi-resistant to antimicrobials strain of *P. aeruginosa*, even when the solutions are stored for four weeks.

Activated solutions are preparations, the mechanism of action of which is on a fundamentally new level - not chemically, as the usual drugs, but electrochemically. Accessible and not expensive apparatuses and technologies are used to obtain them. Therefore, it can be boldly said, that EAASs

are a promising means of science-based medicine of the future.

Anolytes and catholytes obtained upon activation of water with 0.8% NaCl, as well as with a combination of 0.4% NaCl and 0.4% Na₂CO₃, exhibit high antibacterial activity on the tested multi-resistant strain of *P. aeruginosa*. After storage for 29 days, the electrochemically activated aqueous solutions tested preserved these antimicrobial properties to the maximum extent. Upon potentiation of the anolytes by the addition of 96% ethanol with a final concentration of 1% high bactericidal activity was observed within 2 minutes. The use of catholyte of NaCl to dissolve Virkon S resulted in an increase in its effect compared to its use as an aqueous solution.

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