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IMPEDIMETRIC TEST FOR RAPID DETERMINATION OF PERFORMIC ACID (PFA) BIOCIDAL ACTIVITY TOWARD *ECHERICHIA COLI*

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ABSTRACT

Background. Performic acid has recently become available on a commercial scale for potential use in wastewater disinfection and can become an innovative biocide for various purposes in food processing. The aim of our study was: 1) to investigate the antimicrobial resistance of performic acid as high active and non toxic chemical disinfectant against *Escherichi coli* (hygiene indicator test microorganism used in industrial microbiology) and 2) to evaluate the electrical impedance measurement method usefulness for fast and high precise test of antibacterial activity.
Material and methods. Four types of antimicrobial disinfectants (commercial 35% hydrogen peroxide, 1% performic acid, 35% hydrogen peroxide and 15% formic acid) were tested against *Escherichia coli as* hygiene indicator test microorganism. By evaluating the biocidal activity of selected disinfectants two methods were compared: electrical impedance measurement and classical serial dilution method with turbidity effect.
Results. It was stated that the performic acid expressed the highest antibacterial activity in comparison to other tested peroxide disinfectants: commercial 35% hydrogen peroxide solution and components required for performic acid production: 35% hydrogen peroxide solution with stabilizers and 15% formic acid solution with stabilizers). It was demonstrated that the proposed alternative microbiology method of electrical impedance measurement facilitates a rapidly and more precise analyses of the intensity of disinfectants inhibition effect.
Conclusions. It can be postulated that both, the performic acid disinfectants as well as the impedimetric method can be a good advantage in the industrial microbiology.

Key words: performic acid, impedance measurement, disinfection, biocides

INTRODUCTION

Performic acid (CAS registry number and name: 107-32-4, methaneperoxoic acid) is a mixture of two components: solution of hydrogen peroxide (35% solution of H_2O_2) and formic acid (concentrations from 10 to 20%) with stabilizing substances, mixed in the ratio 1:1. So far research concerning the influence of peroxide acids on microorganisms has been carried out in different aspects. It was assumed that those compounds have a stronger effect on airborne microbes that other peroxides and oxidative substances. Their disinfective activity is associated with release of active oxygen [Baldry et al. 1991, Lefevre et al. 1992]. Probably trioxide binding, as well as thiol groups which can be found in enzymes, structural proteins and other building

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compounds are oxidized and destroyed. The peroxide acids activate for example catalase – enzyme known for detox of free radicals [Lefevre et al. 1992, Vissers et al. 2009]. Double bonds are also oxidized. Acid, which penetrates into the inferior of a cell impedes the basic metabolic traces and the active transport through membranes. The compound has also the ability to affect DNA [Baldry et al. 1991, Lefevre et al. 1992].

Performic acid (PFA) is also one of the peroxide compounds, and can be treated as an alternative to other bactericidal substances, e.g. for the peracetic acid [Gehr et al. 2009]. Performic acid and peracetic acid are rather similar disinfectant compounds. The efficiency of both these compounds base on active oxygen operation [Rutala and Weber 2001]. Performic acid has recently become available on a commercial scale for potential use in wastewater disinfection [Gehr et al. 2009, Vissers et al. 2009]. Furthermore a wide potential of PFA as an innovative biocide for various purposes in food processing related applications was already discussed by Dobrucka et al. [2009]. The substance inactivates viruses, bacteria and bacterial spores, bacilli and microscopic fungus and exhibit high antibacterial activity also in low temperatures [McWilliam and Stewart 2002, Heinonen-Tanski and Miettinen 2010].

In the present study the antibacterial activity of performic acid was evaluated by employing the electrical impedance measurement method – an alternative for the classical serial dilution method. The principle of the impedimetric technique is to measure electrical impedance changes caused by the microbial activity during growth in the culture medium. Molecules of high molecular weight present in the medium (e.g. proteins, carbohydrates and fats) are broken down by viable microorganisms to ionized metabolic products. Smaller, charged molecules result in an increase in electric current conductivity and consequently lead to a decrease in the electrical impedance of the culture environment [Gomez et al. 2002, Noble 1999, Yunus et al. 2002, Nowak et al. 2005]. In general, this method is used for rapid detection or quality and quantity analyses of microorganisms, especially in the quality control of food, pharmaceuticals and cosmetics [Wawerla et al. 1999, Moldenhauer 2003]. However, attempts have been made to employ impedance measurements to characterize the stimulating or inhibiting effect on the metabolic activities of microorganisms. Curda and Plockova [1995] investigated inhibiting properties of honey and its influence on the metabolic activities of lactic acid bacteria, while Paquet et al. [2000] applied the measurement of electrical impedance to assess the activity of starter cultures in the process of cheese production. Whereas Lasik et al. [2002] and Nowak et al. [2005] used the impedance technique to evaluate the inhibition effect of high loaded food industry wastewater on single and mixed bacteria populations growth kinetic.

In the present study two main research aims were stated. The first aim was to investigate the antimicrobial resistance of performic acid as high active and non toxic chemical disinfectant against *Escherichi coli*. This bacterium was chosen in our experiment as hygiene indicator test microorganism used in industrial microbiology. And the other aim of the study was to evaluate the possibility of electrical impedance measurement method used for fast and high precision test of antibacterial activity. The proposed alternative microbiology method was compared to classical serial dilution method. The parameters of minimal ihhibitory concentration and minimal bactericidal concentration (as results of classical method) were confronted with impedance changes profile and impedance detection time (as result of impedimetric method).

MATERIAL AND METHODS

Disinfectant agents

Four types of antimicrobial disinfectant agents were tested: A. commercial 35% hydrogen peroxide solution, B. 1% performic acid solution and components required for its production: C. 35% hydrogen peroxide solution with stabilizers and D. 15% formic acid solution with stabilizers. 1% performic acid solution was prepared by mixing 35% hydrogen peroxide solution (C) and 15% formic acid solution (D), in the ratio 1:1.

Microorganisms

The strain of *Escherichia coli* PCM 318 O2:K1(L):H4 from a strain collection of the Institute of Immunology and Experimental Therapy in Wrocław, Poland was used as the test microorganism in the performed investigations. The test bacteria were cultured and tested using a nutritional broth (BTL, Poland) as growth medium at 37°C for 48 h.

Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) is assumed to be the lowest concentration, at which no microbial growth was observed [Wiegant et al. 2008, Mongalo et al. 2013, Sun at al. 2012]. Two-fold serial dilutions of the four tested disinfectant agents were prepared for their antibacterial resistance evaluation. The serial dilutions were prepared in test tubes containing the bacteria growth medium and tested disinfectant agents in a certain concentration. The lowest concentration of the tested agents that inhibited the visible growth of *E. coli* after 48 h incubation was determined by turbidity observations and was specified as MIC. The inoculation of the hygiene indicator bacteria *E. coli* was applied in the volume of 5% v/v.

Determination of minimal bactericidal concentration (MBC)

The antibacterial compound at a concentration equivalent to the minimal inhibitory concentration (MIC) does not always completely inactivate microorganisms, as sometimes it only inhibits their growth. The determination of bactericidal activity was the next step in this study. The minimal bactericidal concentration (MBC) defines the lowest concentration of a disinfectant agent, which exhibits the ability to kill the bacteria cell [Robinson et al. 1985]. In order to verify whether the tested preparation only inhibits the bacteria growth (bacteriostatic activity), or it inactivates them totally (bactericidal activity), an additional passaging of the tested bacteria culture was performed. From test tubes containing the disinfectant agent in the concentrations at which microbial growth inhibition was observed (MIC), the culture was passaged into a broth medium with no addition of the tested preparation. Lack of turbidity effect attest to the bactericidal activity in the tested concentration while the visible growth evidenced that the tested agent in this concentration exhibit only bacteriostatic activity.

Principle of electrical impedance changes measurement

The performic acid inhibitory effect was also evaluated by analysing the electrical impedance changes which occurred in the growth medium. Impedance changes, caused by metabolic activity of the tested bacteria during growth in the media contained tested disinfectants agents were measured in the Automatic Analyzer of Microorganisms Growth, BacTrac 4100 (Sy-Lab, Austria). Special 10-ml measuring test tubes (Sy-Lab) equipped with 4 electrodes were used in the experiment. The test tubes were filled with 9 ml of growth medium containing the antimicrobial agent, and then inoculated with 1 ml inoculum of the tested bacteria. The measuring test tubes were incubated at 37°C in a thermostat of the Automatic Analyzer of Microorganisms Growth. Changes in the electrical impedance were calculated in relation to the initial value according to the following formula:

$$y = \frac{(y_0 - y_i)}{y_0} \cdot 100\%$$

where:

y – changes in electrical impedance of growth medium,

y_0 – value of electrical impedance at the beginning of culturing,

y_i – value of electrical impedance at a given point of measurement.

Changes of electrical impedance caused by metabolic processes of bacteria were presented in the form of impedance changes curve, which graphical picture is parallel to the classical microbial growth curve (with the lag-, logarytmic- and stationary-phase) [Gomez et al. 2002, Noble 1999, Paquet et al. 2000, Wawerla et al. 1999, Ben-Yoav et al. 2011, Dweik et al. 2012]. For the purpose of a comparative analysis of the obtained curves, the parameter of impedance detection time (IDT) was determined. The IDT parameter was established when the stationary phase began (at 2% changes of medium impedance).

RESULTS

The preliminary experiment made it possible to characterise bactericidal and bacteriostatic properties of the four tested preparations: A. commercial 35% hydrogen peroxide solution, B. 1% performic acid solution and components required for its production: C. 35% hydrogen peroxide solution with stabilizers and D. 15% formic acid solution with stabilizers. It was attempted to determine parameters of minimal inhibitory concentration (MIC) and minimal

Table 1. Growth characteristics of the tested bacteria *E. coli* PCM 318 O2:K1(L):H4 during growth in medium containing the tested disinfectants

Dose of disinfectant agent % v/v	A commercial 35% hydrogen peroxide		B 1% PFA		C 35% hydrogen peroxide solution with stabilizers		D 15% formic acid solution with stabilizers	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1.56	–	–	–	–	–	–	–	–
0.78	–	–	–	–	–	–	–	–
0.39	–	–	–	–	–	–	–	±
0.195	–	–	–	–	–	–	–	±
0.097	–	–	–	–	–	–	+	+
0.048	–	–	–	–	–	–	+	+
0.024	–	+	–	+	–	+	+	+
0.012	±	+	±	+	±	+	+	+
0.006	+	+	+	+	+	+	+	+

+ – bacteria growth – high turbidity, ± – inhibited growth, weak turbidity, – – no growth (lack of turbidity).

bactericidal concentration (MBC). According to the serial dilution tests three of the evaluated disinfectants agents: A. commercial 35% hydrogen peroxide solution, B. 1% performic acid solution and C. 35% hydrogen peroxide solution with stabilizers, demonstrated the highest antibacterial activity. The minimal inhibitory concentration was observed by 0.024% v/v dosage and minimal bactericidal concentration by 0.049% v/v dosage (Table 1, 2). No significant differences ($p < 0.05$) between the three disinfectants were found. Clearly weaker activity demonstrated 15% formic acid solution with stabilizers. The minimal inhibitory concentration, as well as the minimal bactericidal concentration, were significantly higher and reached 0.195 and 0.78% v/v respectively (Table 2).

In the next stage of the study changes, in electrical impedance of the culture media, were analysed. When analysing the course of changes in medium electrical impedance during growth of test bacteria in the presence of analysed disinfectants it was observed that all the tested preparations significantly inhibited growth, as well as metabolic activity of the tested microorganisms. The values of minimal inhibitory concentrations and minimal bactericidal concentrations determined by serial dilution method were confirmed by electrical impedance measurements. Again the three disinfectants: commercial 35% hydrogen peroxide solution (A), 1% performic acid solution (B) and 35% hydrogen peroxide solution with stabilizers (C) expressed the highest inhibitory effect while 15% formic acid solution with stabilizers (D) showed the antibacterial

Table 2. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the tested bactericidal agents towards the tested bacteria *E. coli* PCM 318 O2:K1(L):H4

Tested bactericidal agents	MIC % v/v	MBC % v/v
A. Commercial 35% hydrogen peroxide	0.024	0.049
B. 1% PFA	0.024	0.049
C. 35% hydrogen peroxide solution with stabilizers	0.024	0.049
D. 15% formic acid solution with stabilizers	0.195	0.78

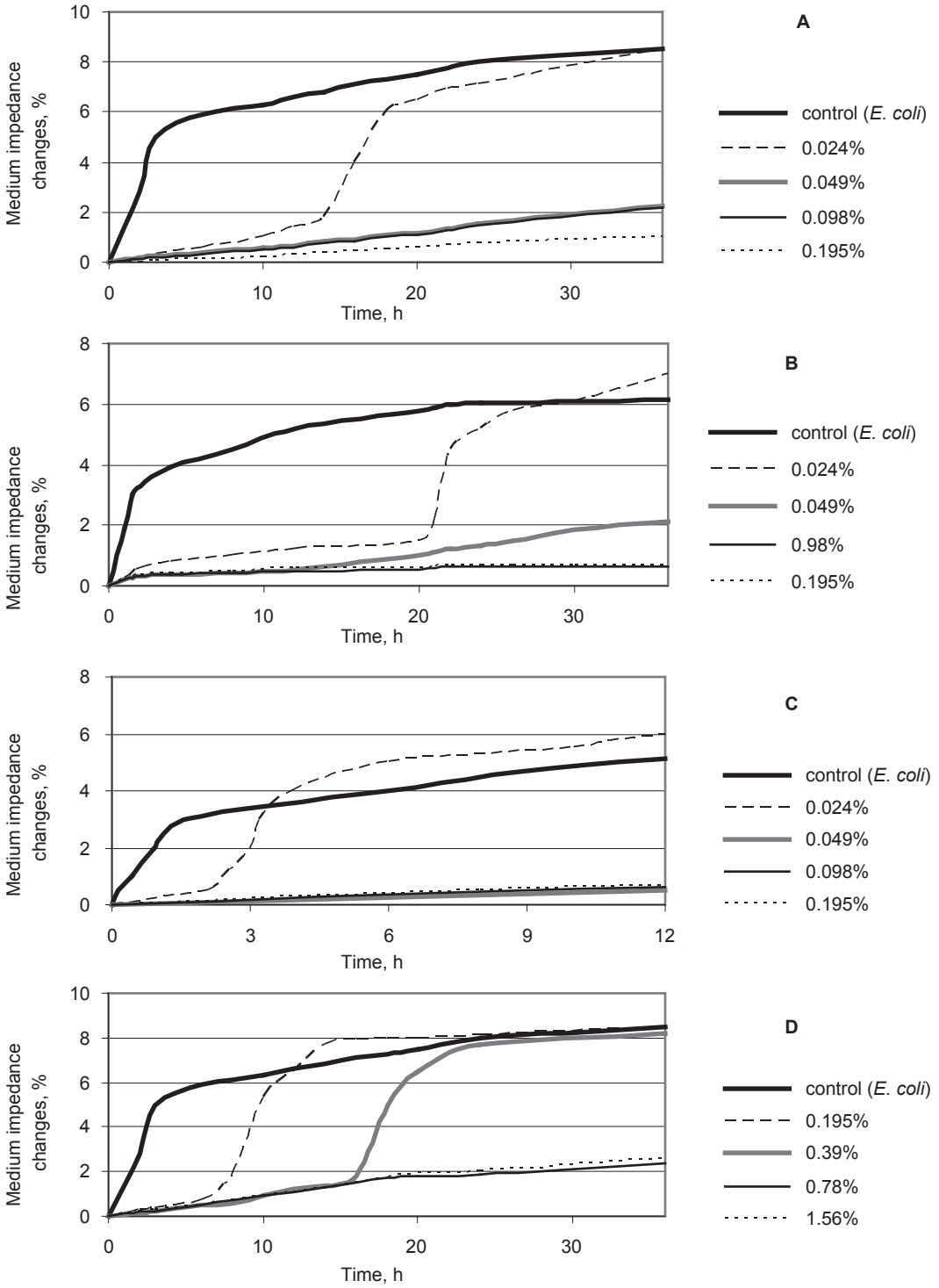

Fig. 1. Changes of medium electrical impedance during cultivation of *E. coli* with the tested disinfectants: A. commercial 35% hydrogen peroxide solution, B. 1% performic acid solution and components required for its production, C. 35% hydrogen peroxide solution with stabilizers, D. 15% formic acid solution with stabilizers

Table 3. Parameters of impedance detection time determined during incubation of *E. coli* PCM 318 O2:K1(L):H4 in the growth medium with the tested disinfectants

Dose of disinfectant agent % v/v	Impedance detection time, h			
	A	B	C	D
1.56	–	–	–	no growth
0.78	–	–	–	no growth
0.39	–	–	–	16.75 ±0.29
0.195	no growth	no growth	no growth	8.12 ±0.16
0.098	no growth	no growth	no growth	–
0.049	no growth	no growth	no growth	–
0.024	14.7 ±0.12	20.77 ±0.22	3.12 ±0.09	–
Control (*E. coli*)	0.43 ±0.03	0.43 ±0.03	0.43 ±0.03	0.43 ±0.03

– – not analysed.
no growth – for the curves where no stationary phase appeared.

activity only in the significantly higher concentrations (Fig. 1, Table 2).

However, in the performed experiments it was observed that from the three disinfectants, which did not differ significantly according to the classical serial dilution method, 1% performic acid solution expressed the highest ($p < 0.05$) antibacterial activity. The impedance detection time was deleted to 20.77 ±0.22 h in comparison to *E. coli* control growth (only growth medium) where the impedance detection time was 0.43 h (Table 3). Therefore, from the three most active disinfectants, 1% performic acid solution was found as the one with the highest antibacterial potential. 35% hydrogen peroxide solution (A) as well as 35% hydrogen peroxide solution with stabilizers (C) presented significantly lower inhibition ability, deleting the impedance detection time until 14.7 and 3.12 h respectively (Fig. 1, Table 2).

DISCUSSION

Microbiological indicators are bacteria shown to be associated with disease-causing organisms. WHO states, that the presence of *E. coli* always indicates potentially dangerous contamination requiring immediate attention [Knaflewska and Pospiech 2007, El-Hofi et al. 2010, Centres... 2012, Grujić et al. 2013]. Thus due to its high prevalence and disease-causing properties, *E. coli* was chosen for this experiment as microbiological hygiene indicator.

It was proposed in this study to apply measurements of electrical impedance as a rapid and precise test assessing antimicrobial properties of a performic acid preparation. The method is currently applied mainly for the purpose of rapid detection of microorganisms [Gomez et al. 2002, Noble 1999, Ben-Yoav et al. 2011, Dweik et al. 2012]. However, it also facilitates an analysis of the inhibitory action of different preparations used in hygienisation [Wawerla et al. 1999, Moldenhauer 2003, Otles and Yalcin 2012]. In the conducted experiment, changes in the resistance of alternating current in the culture medium were followed, as occurring under the influence of growth of selected microorganisms in the medium containing tested disinfectants. After 48 h incubation at a temperature of 37°C curves of impedance changes registered during the tested bacteria growth were analysed. Typical curves of bacterial growth were graphic images of these changes (Fig. 1). In the general concept of this method, when applying disinfectants of inhibitory

character the lag-phase period (registered as impedance detection time) is extended in comparison to the control sample (bacteria growing in the medium without disinfectant). This indicates the inhibition effect of the tested disinfectant towards the tested microorganism [Curda and Plockova 1995, Gomez et al. 2002, Wawerla et al. 1999, Kim et al. 2012, Qi et al. 2013]. Thus when increasing doses of the preparation the impedance detection time is gradually extended. At higher doses of agents with a bactericidal activity against the test bacteria a complete inhibition of growth is found for these microorganisms [Lasik et al. 2002, Moldenhauer 2003].

In the light of the results presented above, it was stated that application of the impedance measurement proposed in the presented study allow faster and more accurate analyse of the inhibitory effect. The method of electrical impedance changes measurement makes it possible to precisely analyze of the intensity of inhibition effect in contrast to the conventional serial dilution method (MIC and MBC). In the classical dilution method only those concentrations are detected, at which growth is no longer observed within a specified period of time. For instance the application of 0.024% v/v of performic acid resulted complete lack of growth within 48 h incubation; when applying lower concentrations: 0.012% v/v a weak growth of test bacteria was observed (Table 1). The impedimetric method provide observations and analyses of dynamics of the investigated inhibition process. Lack of growth was also observed at a concentration of 0.024% v/v but only until about 20 h incubation, after this time a significant extension of the lag phase period was found, which indicates a significant, although incomplete growth inhibition at this concentration (Fig. 1). In such case there is a possibility to evaluate the time needed for bacteria for adaptation in the inconvenient environment. It is possible to reach this precise knowledge only when analysing the electrical impedance signals (Fig. 1) no when classical serial dilution method is used and turbidity effect is determined.

In view of the recorded results it may be concluded that the performic acid exhibits high antibacterial activity against *E. coli* bacteria. The mechanism of biocidal action of performic acid most probably results from the presence of hydroxyl radicals, which causes irreversible damage in the reduced functional groups of proteins and thus destroys the enzymatic apparatus and structure of cells causing among others permeability of cell membranes [Sun and Smith 1988, Gehr et al. 2009, Kuo and Abustan 2009, Vissers et al. 2009]. Performic acid exhibits a relatively non-specific mechanism of action. Additionally, performic acid molecule contains only one carbon atom what makes this disinfectant very interesting. It degrades rapidly (until 8 hours) and its degradation products: formic acid, oxygen and water make less organic residues than other commercial disinfectants.

CONCLUSION

The results presented let us conclude that 1) the proposed method of electrical impedance changes measurement facilitate a rapid and more precise analysis of the intensity of disinfectants inhibition effect in comparison to classical serial dilution method and 2) the performic acid can be a good alternative as non toxic disinfectant agent with a high antibacterial activity.

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TESTY IMPEDYMETRYCZNE STOSOWANE W CELU SZYBKIEJ OCENY PRZECIWDROBNOUSTROJOWEJ AKTYWNOŚCI KWASU NADMRÓWKOWEGO WOBEC BAKTERII *ECHERICHIA COLI*

STRESZCZENIE

Wprowadzenie. Od niedawna kwas nadmrówkowy stał się preparatem dostępnym i stosowanym w skali przemysłowej do dezynfekcji ścieków, może również okazać się innowacyjnym biocydem stosowanym w szeroko pojętym przetwórstwie żywności. Celem prezentowanych badań była: 1) ocena antymikrobiologicznej aktywności kwasu nadmrówkowego jako nietoksycznego chemicznego dezynfektanta wobec *Escherichi coli* (mikroorganizmu testowego stosowanego jako wskaźnik higieniczny w mikrobiologii przemysłowej) oraz 2) ocena przydatności metody pomiarów zmian impedancji elektrycznej do szybkich i specyficznych testów aktywności antybakteryjnej.

Materiał i metody. Cztery rodzaje środków dezynfekujących (komercyjny 35-procentowy nadtlenek wodoru, 1-procentowy kwas nadmrówkowy, 35-procentowy nadtlenek wodoru i 15-procentowy kwas mrówkowy) zostały przetestowane wobec bakterii *Escherichia coli* jako mikroorganizmu wskaźnikowego stosowanego w testach higienicznych. Do oceny antydrobnoustrojowych właściwości wybranych dezynfektantów zastosowano dwie metody: metodę pomiaru zmian impedancji oraz klasyczną metodę seryjnych rozcieńczeń z oceną zmian zmętnienia testowanej hodowli.

Wyniki. Stwierdzono, że kwas nadmrówkowy wykazywał największą aktywność antybakteryjną w porównaniu z pozostałymi przetestowanymi dezynfektantami: komercyjnym 35-procentowym nadtlenkiem wodoru oraz komponentami niezbędnymi do syntezy kwasu nadmrówkowego: 35-procentowym nadtlenkiem wodoru i 15-procentowym kwasem mrówkowym wraz ze stabilizatorami. Wykazano, że zaproponowana alternatywna metoda mikrobiologiczna – pomiar impedancji elektrycznej – pozwoliła na szybszą i precyzyjniejszą analizę intensywności właściwości inhibujących dezynfektantów.

Wnioski. Stwierdzono, że zarówno kwas nadmrówkowy jako dezynfektant, jak i metoda impedymetryczna mogą być z korzyścią wykorzystywane w mikrobiologii przemysłowej.

Słowa kluczowe: kwas nadmrówkowy, pomiary impedymetryczne, dezynfekcja, biocydy

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