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Research Article

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Inhibiting Effect of Electrochemically Activated Aqueous Solutions on Growth Biofilms

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ABSTRACT

This study assessed the impact of the electrochemically activated aqueous solutions (ECA AS) on the growth and formation of the biofilm of lactic acid bacteria (LAB). The effectiveness of biocides produced by the unipolar electrochemical activation of aqueous solutions of sodium chloride was studied. It was found that sequential treatment of the biofilm with catholyte and anolyte (fractions ECA AS) lead to the most pronounced decrease in the growth rate and density of bacteria. The results obtained demonstrate antibacterial efficacy and the possibility of using ECA AS for the prevention and disinfection of aquatic systems, for example, at the enterprises of the agro-industry and the food industry.

Key words: *Biofilm, Lactic Acid Bacteria, Electrochemically Activated Aqueous Solution, Anolyte, Catholyte, Microbiological Safety, Antimicrobial Treatment, Agro-Industry, Food Industry.*

INTRODUCTION

The close attention paid to biofilms is due to the prevalence of this form of microbial symbiosis both in the natural environment and in industrial systems [1, 2]. Most microorganisms exist in the composition of the biofilm and only about 10% in planktonic form [3, 4]. Biofilms are usually formed on a solid substrate, and represent a community of microorganisms of different species. The structure of the biofilm and the physicochemical properties of its matrix protect microorganisms from the influence of external aggressive factors (pH, presence of reactive oxygen species and mechanical effect of the flow, disinfecting procedures), ensuring aggressive population of new ecological niches.

Environmental factors and the properties of the cells affect the process of biofilm formation. The most important environmental factors are pH, salinity, temperature, osmolarity, oxygen partial pressure, accessibility to nutrient sources, surface properties (of both bacteria and substrate) and the force and type of liquid motion relative to this surface [5-7]. Biofilm cells differ from planktonic cells in gene expression, protein production and resistance to the immune system and antimicrobial agents [8]. This adaptive response depends on the surrounding fluid hydrodynamic conditions which will dictate shear forces and mass transference (oxygen, nutrients, cellular products, etc.). Thus, the biofilm architecture (thickness, porosity, etc.) must be adapted in order to resist shear forces and allow a better access to nutrients and oxygen.

Biofilms pose a problem for the food industry, contaminating equipment, deteriorating the presentation and quality of food through bioconversion [5, 6], contaminating food contact surfaces [7-9]. At enterprises with water processing lines, due to biofouling, there are problems with cleaning and disinfecting the internal surface of pipelines, and therefore with the quality of incoming water [1, 7-9]. In wastewater treatment technologies

using membrane filtration, the biofilm that forms on the membrane clogs the filters, reducing their productivity and efficiency [10]. Biofilm contributes to the development of corrosion of metal surfaces of equipment, and creates the additional difficulty of cleaning surfaces from various materials with roughness and cracks [11-13].

In many industries of biofilm production, complicating the cleaning of technological lines and reducing the effectiveness of their disinfection, cause corrosion and damage to production capacity [14]. Bacteria in the composition of biofilms become more resistant to antibiotics and antimicrobial agents than the cells of the same species in planktonic form [15, 16]. Increasing resistance requires a significant increase in the concentration of disinfectant solutions, which leads to an additional environmental burden on the environment, as well as the higher cost of the final product. Therefore, the development of fundamentally new methods of biofilm removal, which would be both economical, efficient and environmentally friendly, is urgent. In food processing technologies, biocides in the form of ECA AS can be an environmentally friendly and effective method of disinfection [17-19]. ECA AS are classified as safe broad-spectrum disinfectants. Antimicrobial ECA solution (Anolyte - one of the ECA AS fractions), provides the appearance of active short-lived particles that violate the vital biological processes of microorganisms of all types and forms (bacteria, viruses, fungi, and spore forms of microorganisms).

In the last decade, the idea has been discussed in the literature that in the presence of reactive oxygen species (ROS), the lethality of various antimicrobials increases. Experimental data confirming this hypothesis were presented in [20, 21]. The electrochemically activated anolyte contains, in addition to hypochlorous acid, small amounts of metastable impurities of hypochlorite ion, hydrogen peroxide, ozone, and various free radical, ionized reactive oxygen species [17]. As a result of the effects of the anolyte on the biofilm, in addition to the direct action of metastable compounds, the electrostatic equilibrium of the biofilm occurs. This ultimately determines its destruction [22]. In [22], the anolyte is explained by the action of hypochlorous acid on the membrane of a bacterial cell, which provides an osmotic barrier and transmembrane transport of substances.

Due to their physicochemical metastable state, microbial communities do not develop resistance mechanisms to this effect. Anolyte, one of the ECA AS fractions, is used for chemical protection of agricultural products and food industry facilities [23]. Directional saturation of the solution with a complex of metastable chemical compounds, which are formed during electrochemical activation, belong to the category of "green" technologies that do not cause damage to the environment.

MATERIALS AND METHODS

The main object of this work was to study the effect of ECA AS on the cellular component in the processing of biofilms. The experiment was conducted on a biofilm formed by a suspension of microorganisms, which included a complex of LAB (Table 1) and *Escherichia coli (E. coli*).

Table 1. Strains of lactic acid microorganisms* and conditions for their cultivation

Lactic acid strains microorganism	Optimum growth temperature	Cultivation medium
Lactococcus lactis	28-32ºC	Cow's milk, normalized or skimmed
Streptococcus thermophilus	40-45ºC	
Lactobacillus acidophilus	37-39ºC	
Lactobacillus helveticus	40-44ºC	
Propionibacterium freudenreichii ssp. shermanii	30-37ºC	

* - to obtain planktonic form of LAB, water suspension of the freeze-dried starter was used "Evitalia".

The initial suspension is associated with microorganisms, taking into account the bacterial mass of 4×10^9 CFU / cm^3, which is activated by introducing vials of dry ferments in 0.25 l of normalized milk with a temperature of 40 °- 43 ° C. The bacterial culture was diluted with sterilized water to a total volume of 0.75 l, which served as the initiator of biofilm formation, which was grown at room temperature (23 ° C) on glass coupons in a flow reactor for 6 days. Samples pre-washed with running water or ECA AS were prepared for microbiological analysis and light microscopy.

For comparison, the film was treated in a stream of 10% NaOH solution or the ECA AS fraction (Table 2). In the latter case, an alkaline catholyte was taken that contained 10% NaOH saturated with hydrogen with the oxidation reduction potential ORP = -600 mV and anolyte (a mixture of chlorine-oxygen and hydroperoxide oxidants at a concentration of 500 mg / l in equivalent active chlorine). A sample washed with water served as a control.

Table 2. The methods of processing the bacterial film formed on the surface of the glass

Sample	Treatment	time of processing
control (№1)	flow water	1 hour
Sample №2	flow of 10% NaOH solutions	1 hour
Sample №3	flow catholyte	1 hour
Sample №4	flow catholyte and anolyte	1 hour

RESULTS AND DISCUSSION

The effect of processing parameters (anolyte and catholyte content, exposure time) was evaluated by reducing the number of microbial colonies (CFU / cm^3). The results of microscopic analysis (Fig. 1) revealed marked differences in the structure of the biofilm of the control and experimental samples.

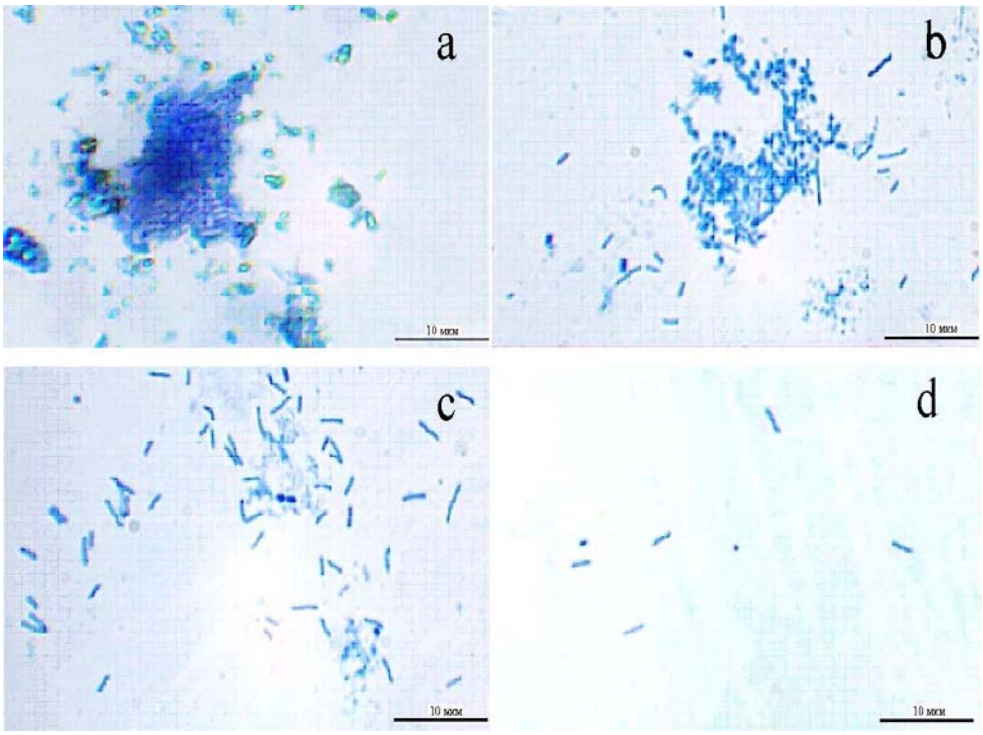

Figure 1: Micrographs of a biofilm formed by LAB. Image of the bacterial film: (a) the preparation was washed with water - control, (b) the preparation was washed with 10% aqueous NaOH solution, (c) the preparation was washed with catholyte, (d) the preparation was washed successively with catholyte and anolyte (g). The size of the division deposited on the image corresponds to 10 microns.

It can be seen (Fig. 1a) that ordinary water does not affect the structure of the biofilm. The loosening of the bacterial film is observed in a stream of 10% NaOH solution (Fig. 1b). As a result of treatment with the catholyte, the matrix of the biofilm and the partially cellular component were removed (Fig. 1c). The complete disintegration of the biofilm is registered after the action by the catholyte in combination with the anolyte, but in this case, rare fragments of cells remain on the surface (Fig. 1d).

Note that traces of the matrix or cellular material on the surface serve as an attractor for the regeneration of biofilms. Additionally, it was also observed that the formed biofilm acts as a constant reservoir of cells that after detaching (due to the flow shear in process of treatment) are able to occupy new surfaces very quickly. Another processing factor may be the change in the physicochemical and mechanical properties of the surface layer of the substrate under the influence of ECA AS. Since the stages of development and existence of biofilms are influenced by transport processes of nutrient transfer and interaction with the fluid medium, which is not only a source of nutrients, but also regulates the transfer of cells that have a direct impact on cell adhesion and biofilm formation. Therefore, we studied the removal of a biofilm formed again on the surface of a glass coupon, which was previously exposed to ECA AS (Fig. 2).

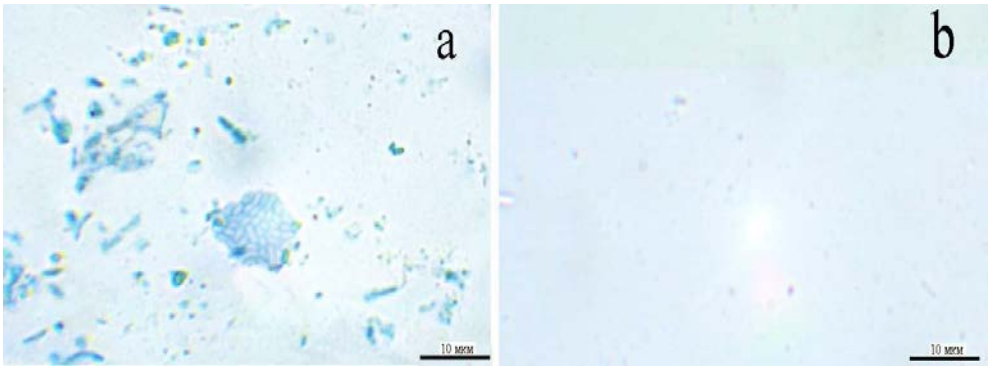

Figure 2: Micrographs of a biofilm formed by lactic acid bacteria on the surface of a glass coupon: (a) repeated on the surface, which has already received the primary biofilm; The size of the division deposited on the image corresponds to 10 microns.

The growth conditions of the biofilm and its processing remained constant throughout the experiment. It can be seen (Fig. 2a) that biofilms are formed on the surface of the coupon previously processed by ECA AS. A visual comparison shows the absence of obvious sources of contamination after the repeated disintegration procedure (Fig. 2b). Second exposure to ECA AS leads to more dense biofilms, which, in turn, reduces the diffusion of molecules within biofilms. And the flow velocity contributes to stronger shear forces, which can contribute to the breakage or detachment of biofilms. Thus, physical and chemical properties ECA AS and mechanical properties of the flow remains effective at killing the secondary biofilm. Both ECA AS fractions must be combined to more completely remove the matrix.

At the next stage, the samples obtained in the laboratory circulation reactor, on the inner surface of the pipeline wall of which a biofilm was formed, were investigated. At the next stage, the samples obtained in the laboratory circulation reactor, on the inner surface of the pipeline wall of which a biofilm was formed, were investigated.

After treatment, the tube was transferred into preparations physiological medium (0, 9% NaCl solution) for subsequent rapid analysis of total viable content by counting the number of viable cells. To do this, on the test plates Petrifilm RAC raised the top sheet and 1 ml of the sample was applied to the surface. Then the top sheet was gently lowered, applying pressure to the plates in order to evenly distribute the liquid sample over the entire surface [24]. The preparation applied to the surface of the Petrifilm RAC plate was incubated for 48 hours at 36 ° C.

On two plates, Petrifilm from one dilution was counted the number of colonies, after which the data were averaged. The mean values were used to quantify the index (BD) of biofilm microbiological density, which was calculated from equation (1) [25].

$$BD = \log_{10}[(CFU /V)^x(v/S)^xK] \quad (1)$$

Where CFU is the number of colonies per cm^2, V is the sample volume in ml, v is the volume of the tube in ml, S is the coupon area in cm^2, K is the dilution factor.

For clarity, the value of the microbiological density of the biofilm obtained for different methods of its processing is shown in the Figure 3.

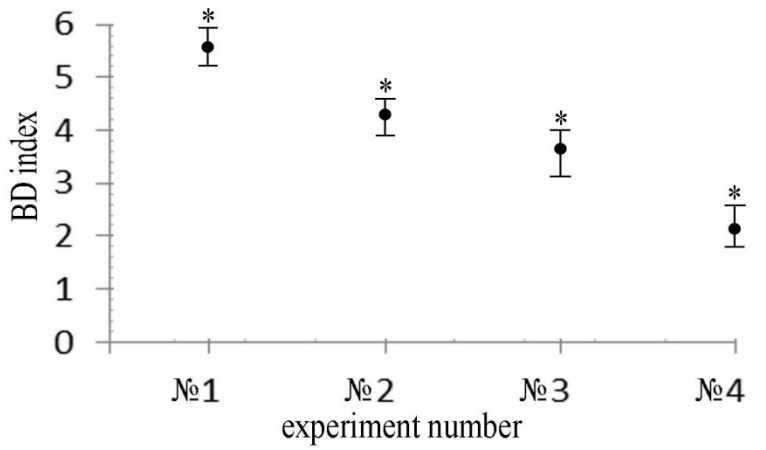

Figure 3. The index (BD) of biofilm microbiological density, where each circle or bar represents the median and variability between replicates of three to five independent measurements, asterisks indicate significant difference (P<0.05) from other experimental groups. Experiments: №1 – treatment with water flow, №2 - treatment with 10% aqueous NaOH solution flow, №3 - treatment with catholyte flow, №4 - treatment with catholyte followed by anolyte flow.

To compare the effectiveness of treatment with aqueous solutions of different nature, we used the Log reduction (LR), which is an indicator of the antimicrobial effectiveness of the disinfectant [26]. The value of this parameter is the difference between the value of the BD of the control and experimental samples. In our case, we used BD readings for a bacterial film grown on a glass coupon and processed in different ways. The obtained dependence of BD on LR is shown in Figure 4.

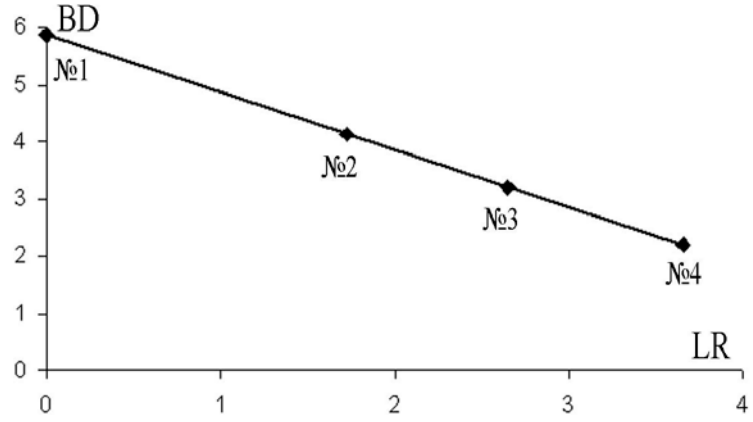

Figure 4. Dependence of BD index on the Log reduction (LR) parameter for a bacterial film formed by *E. coli* and a composition of LAB. Legend: №1 - treatment with water flow, №2 - treatment with 10% aqueous NaOH solution flow, №3 - treatment with catholyte flow, №4 - treatment with catholyte followed by anolyte flow.

The LR parameter demonstrates variability depending on the microbiological density of the BD, which is caused by the method of processing the biofilm. The smallest value (3.66) of LR corresponds to the sequential effect of ECA AS fractions, which means the most effective disintegration of the biofilm. The results obtained are summarized in Table 3.

Table 3. Comparative data of microbiological density and LR parameter obtained for different methods of removing bacterial biofilms

Processing flow	water	10% NaOH	catholyte	catholyte anolyte
Designation processing	№1	№2	№3	№4
BD value *	5,86±0,31	4,14±0,44	3,21±0,43	2,20±0,22
LR value	0	1,72	2,65	3,66

* The data are presented as mean ± standard deviation

CONCLUSION

The obtained experimental results show that the express method for determining microbiological density on Petrifilm RAC plates can be used to assess the quality of removal of a biofilm. In a model experiment with samples of biofilm grown in a reactor, the effectiveness of ECA AS was confirmed on glass coupons and for the inner surface of the reactor tube. Our data demonstrate a significant reduction in bacterial contamination of the surface of the tubes after joint treatment with catholyte and anolyte. Thus, the use of ECA AS, with a wide spectrum of antimicrobial activity and not having a harmful effect on humans after the transition of water to a stationary state, is a promising environmentally friendly direction of disinfection in food and biotechnology.

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REFERENCES

1. Van Houdt R., Michiels C. W. Biofilm formation and the food industry, a focus on the bacterial outer surface // Journal of applied microbiology. 2010, 109 (4), 1117-1131.
2. Srey S., Jahid I. K., Ha S. D. Biofilm formation in food industries: a food safety concern // Food control. 2013, 31(2), 572-585.
3. Roy A. B., Petrova O. E., Sauer K. The phosphodiesterase DipA (PA5017) is essential for Pseudomonas aeruginosa biofilm dispersion // Journal of bacteriology. 2012, C. JB. 05346-11.
4. Shi X., Zhu X. Biofilm formation and food safety in food industries // Trends in Food Science & Technology. 2009, 20(9), 407-413.
5. Suvorov O. A., Volozhaninova S. Yu., Pugachev I. O., Kanina N. Yu., Voyno L. I., Yudina T. P. Provision of Microbiological Safety in The Food Industry Based on Special Technological Supporting Solutions. International Journal of Pharmaceutical Research & Allied Sciences, 2018. 7(1): 103-113.
6. Dourou D. et al. Attachment and biofilm formation by Escherichia coli O157: H7 at different temperatures, on various food-contact surfaces encountered in beef processing // International journal of food microbiology. 2011, 149 (3), 262-268.
7. Ignatenko A. V. Izuchenie obrazovaniya bioplenok i bakterij i ocenka ih ustojchivosti k biocidam // Trudy belorusskogo gosudarstvennogo universiteta. Seriya 4, Himiya i tekhnologiya organicheskih veshchestv. – 2008. 4 (1), 173-176.
8. Lührig K., Canbäck B., Paul C.J., Johansson T., Persson K., Rådström P. Bacterial Community Analysis of Drinking Water Biofilms in Southern Sweden // Microbes Environ. 2015. Vol. 30, No. 1, 99-107. https: // www.jstage.jst.go.jp/browse/jsme2 doi:10.1264/jsme2.ME14123
9. Handbook by Lelieveld Handbook of hygiene control in the food industry // Edited by H.L.M. Levieveld, M.A. Mostert, J. Holah. – CRC Press. – Boca Raton Boston, New York, Washington, 2016.
10. Redman J. A., Walker S. L., Elimelech M. Bacterial adhesion and transport in porous media: role of the secondary energy minimum, Environmental Science Technology, 2004. 38 (6), 1777–1785.
11. Doyle R. J. Contribution of the hydrophobic effect to microbial infection // Microbes and Infection 2, 2000 391-400.
12. CHernyavskij V. I. Bakterial'nye bioplenki i infekcii (lekciya) // Annals of Mechnikov Institute. 2013, 1, 86-90.
13. Sirotkin I. V. Sovershenstvovanie sanitarno-mikrobiologicheskogo kontrolya kachestva profilakticheskoj dezinfekcii v cekhah po pererabotke myasa: diss. ... kandidata veterinarnyh nauk. - Moskva, 2015, 144.
14. Simoes M., Simoes L., Vieira M. A review of current and emergent biofilm control strategies // LWT-Food Science and Technology. 2010, 43(4), 573-583.
15. Olsen I. Biofilm-specific antibiotic tolerance and resistance // European Journal of Clinical Microbiology & Infectious Diseases. 2015, 34(5), 877-886.
16. Holmberg A., Rasmussen M. Mature biofilms of Enterococcus faecalis and Enterococcus faecium are highly resistant to antibiotics // Diagnostic microbiology and infectious disease. 2016, 84(1), 19-21.

17. Bakhir V. M. Elektrohimicheskaya aktivaciya: Izobreteniya, tekhnika, tekhnologiya, M.: Viva-Star. 2014, 512.
18. Pinto L., Baruzzi F., Ippolito A. Recent advances to control spoilage microorganisms in washing water of fruits and vegetables: the use of electrolyzed water // III International Symposium on Postharvest Pathology: Using Science to Increase Food Availability. 2015, 379-384.
19. Pogorelova M. A., Kuznetsov A. L., Suvorov O. A. Does Electrochemically Reduced Water Remove Bacterial Film? // International Journal of Pharmaceutical Research and Allied Sciences, 2018. 7(2), 139-142.
20. Hong Y., Drlica, K. Zhao X. Antimicrobial-Mediated Bacterial Suicide // Antimicrobial Resistance in the 21st Century. Pp. 619-642 https://link.springer.com/chapter/10.1007/978-3-319-78538-7_20
21. Luan G., Hong Y., Drlica K., Zhaoa X., Suppression of Reactive Oxygen Species Accumulation Accounts for Paradoxical Bacterial Survival at High Quinolone Concentration // March 2018, 62(3), https://aac.asm.org/content/62/3/e01622-17
22. Cloete T. E., Thantsha M. S., Maluleke M. R., Kirkpatrick R. The antimicrobial mechanism of electrochemically activated water against Pseudomonas aeruginosa and Escherichia coli as determined by SDS-PAGE analysis // Journal of Applied Microbiology, 379-384.
23. Zon G. A., Vashchik E. V., Ivanovskaya L. B. Effektivnost' rastvorov gipohlorita natriya dlya profilaktiki psevdomonoznoj infekcii v inkubatorii. 2016, 12-17.
24. de Castilho N. P. A. et al. Adequacy of Petrifilm Aerobic Count plates supplemented with de Man, Rogosa & Sharpe broth and chlorophenol red for enumeration of lactic acid bacteria in salami // Meat science. 2015, 110, 253-261.
25. Fritz B. G. et al. Evaluation of Petrifilm Aerobic Count Plates as an Equivalent Alternative to Drop Plating on R2A Agar Plates in a Biofilm Disinfectant Efficacy Test // Current microbiology. 2015, 70(3), 450-456.
26. European Committee for Standardization. Chemical disinfectants and antiseptics-hygienic handrub-test method and requirements (phase2/step2) [European standard EN 1500]. Brussels, Belgium: Central Secretariat. 2013.