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ANTIMICROBIAL ACTIVITY OF NATURAL SOAPS TESTED BY BIOSCREEN METHODOLOGY

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The aim of this study was to combine the utilization of waste frying oils within soap making process in order to make useful and environmentally friendly solutions and development of methods for determination of the antimicrobial effect of those created products. Soaps were made from edible oils which are fried in laboratory conditions. The antimicrobial activity of soaps was done against *Staphylococcus aureus* species as one of the representatives of the human skin microbiome. Two methods were applied: agar dilution method and the method including kinetics following on Bioscreen microbiology reader. In the first method, the number of CFU was followed on agar medium with and without different soap solutions after incubation for 24 hours at 30 °C. The result for IC₅₀ (inhibition concentration for 50% of population) was 0.08 mg/mL. Minimal inhibition concentration was detected at 0.41 mg/mL and minimal bactericidal concentration was observed at > 0.75 mg/mL for selected soap solution. Soap concentrations of 0.3 mg/mL of soaps (made from fresh and fried oil) were used for Bioscreen assessment with measurement on every hour during the 7 hours of incubation at 30 °C. 5-second sequence of shaking of the microplate was applied before each measurement which was done at the wavelength of 610 nm. The growth coefficients of the culture with soap solutions added and from the growth of culture only were compared. The growth of *S. aureus* subjected to soaps made from fresh and fried oils was inhibited 55.3% and

69.7% respectively against the control during the first seven hours of incubation. From results obtained, it was concluded that there is a great potential of the Bioscreen as a method for further studies on antibacterial activity of soaps made from waste frying oils.

Keywords: skin microbiome, staphylococci, frying oils, soap, disinfection, new soap application, Bioscreen

INTRODUCTION

Soaps are the important industrial products for removal and neutralization of harmful and pathogenic microorganisms, which can be colonized on human skin forming biofilm [19]. The structure of such biofilm is complicated and depends on environmental factors and hygiene of each individual. Microbial skin biofilm helps bacteria to adapt to life on the skin surface and create mechanisms for survival of colonies. Creating of biofilms can also lead to creating virulence, pathogenesis and enhanced resistance to antibiotics [7].

The human skin is a habitat of different groups of microbial communities and their disbalance can endanger its protective role [10]. The main microbial genera, which can form biofilms in different parts of the human body are *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Propionibacterium*, *Malassezi*, *Brevibacterium*, *Acinetobacter* and *Dermabacter*. Among them, *Staphylococcus aureus* species was found to be the most common cause of human skin infections [17].

The commercially available hand soaps contain substances like triclosan, which have good antimicrobial properties but are also harmful to the human skin and microbiome [6]. It was also found that triclosan supports the growth of *S. aureus* in the nasal epithelium [21]. Various studies confirmed that there is no significant difference in the effect of antimicrobial soaps in comparison to plain soaps during the process of washing hands. While using antimicrobial soaps there is always a risk for increased resistance of certain microorganisms to the applied antibiotic [3]. Beside of fats and oils that are their constituents some antimicrobial agents like detergents are added in order to increase the power of the soap activity [18].

Frying oils are often used for temperature processing of various foods at high temperatures in the food industry, restaurants and households. About 17 million tons of waste frying oils are produced in the World annually and this production has an increasing trend [5]. The biggest environmental problem is its improper disposal, since it has as a consequence clogging of the drainage systems, harm to the wildlife and much more other [20]. In the process of wastewater treatment oil sticks to apparatuses, causes corrosion of the equipment and lowers the efficiency of wastewater treatment [13].

Today, there is a clear need to think about further use of some used product before its discharge. Reverse logistics systems play important roles in both environmental and economic aspects in the last 10–20 years [15]. The production of soaps is not harmful to nature since there are no by-products formed in its production. Also, it requires a minimal amount of energy used for the process of saponification [14]. In the process of saponification, we can also use waste frying oils and utilize it in a way which is harmless to the nature [8]. Nowadays, trends are going toward the direction of production of products of natural origin which does not include the addition of chemical agents. Since there is a great perspective for the production of soaps from waste frying oils, it is of crucial

importance to investigate their antimicrobial and biological effects, including toxicity for microbial skin biofilm (main bacterial genera located on the skin) and eventual possible allergic reactions that these kind of products can cause. All these mentioned problems are still actual for finding perspective methods for utilization of waste frying oils, their application for safe soaps creation for human skin with inhibition effect for pathogenic microorganisms, but not for the commensal microbiome.

The Bioscreen microbiology reader is an instrument which works on the principle of measurement of optical density. While growing, microorganisms are changing the turbidity of the liquid media in which they are present. The instrument software can follow the kinetics and show the results as a change in optical density over time [12]. Readings are done for samples in wells of the microplate which can be thermostated. One microplate has 100 wells and the readings can be done on one or two plates.

The aim of this study was to compare the possibilities of the methods for determination of the antimicrobial activity of newly synthesized soaps made from used frying oils (agar dilution method on Petri dishes with an automated method using Bioscreen microbiology reader to follow the kinetics of the experiment setup).

MATERIALS AND METHODS

The samples of soap were synthesized from different types of oils at the Department of Plant Origin Foodstuffs Hygiene and Technology, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. The oils used for the experiments were olive oil „EL TORO“ from producer Aceites Ybarra from Spain and rapeseed-palm oil (80:20) „FRITO MANKA“ from producer Fabio Product from the Czech Republic. The frying oils were prepared in laboratory conditions by consecutive heating and cooling of fresh samples of mentioned oils.

The soap for purpose of the experiment is made with potassium hydroxide using the cold method and the following recipe: 280 g of oil, 36.04 g and 34.00 g of sodium hydroxide (NaOH) for the olive oil and rapeseed-palm oil soaps respectively and 106.40 g of distilled water. The NaOH and water were mixed in an ice bath and stirred until total dissolving of NaOH. Then the oil was added and mixed until pudding-like consistency was reached. Once a visible trace appeared on the surface, the mixture was poured into a silicone mould and smoothed with a glass plate. After 24 hours, the soap was removed from the mould and transferred to a filter paper for maturing.

Staphylococcus aureus SA812 was used in this study. This bacterial strain was kept in Czech Collection of Microorganisms (CCM) at the Department of Experimental Biology, Faculty of Science at Masaryk University in Brno, Czech Republic. The culture of *S. aureus* SA812 was diluted to 10% solution in nutrient broth, HiMedia, India (peptone 5 g, sodium chloride 5 g, beef extract 1.5 g, yeast extract 1.5 g) and incubated for 24 hours at 30 °C, after that, it was used for experiments.

The antimicrobial activity of the samples was done using the agar dilution method (Fig. 1) and determination of minimal inhibition concentration (MIC) [9].

The growth of *Staphylococcus* was followed on nutrient agar medium incubated at 30 °C on Petri dishes. Different concentrations of soap solution in quantity of 1 mL were added to the 19 mL of molten and tempered agar, mixed and then poured to the Petri dishes. Everything was done in triplicate together with control (culture incubated on Nutrient agar dishes only). Inhibition was calculated as the percentage of reduction of

colonies on Petri dishes with added soap against the control. The results are presented as IC_{50} (inhibition concentration for 50% of the bacterial population), IC_{90} (inhibition concentration for 90% of the bacterial population which is also called MIC – minimal inhibition concentration), and MBC – minimal bactericidal concentration, above which there is no growth of bacterial colonies.

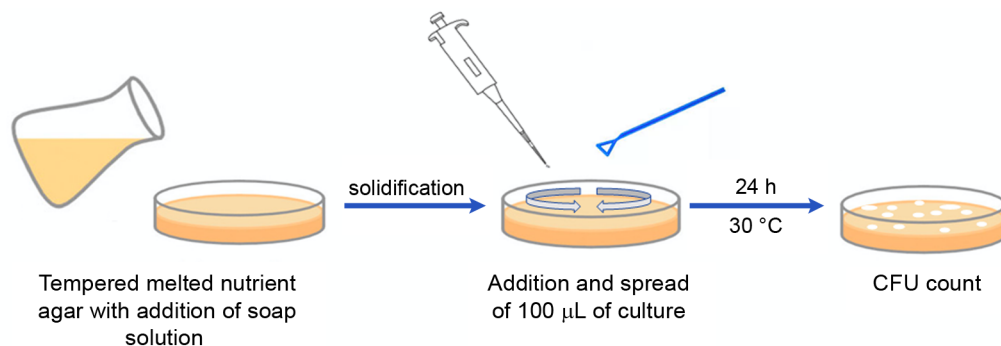


Fig. 1. Process flow in agar dilution method for determination of MIC

For the purpose of the experiment on Bioscreen, total volumes of 300 µL of the sample were used (Fig. 2). For positive control, 30 µL of culture was added to 270 µL of nutrient broth and for negative control 300 µL of Nutrient broth was used.

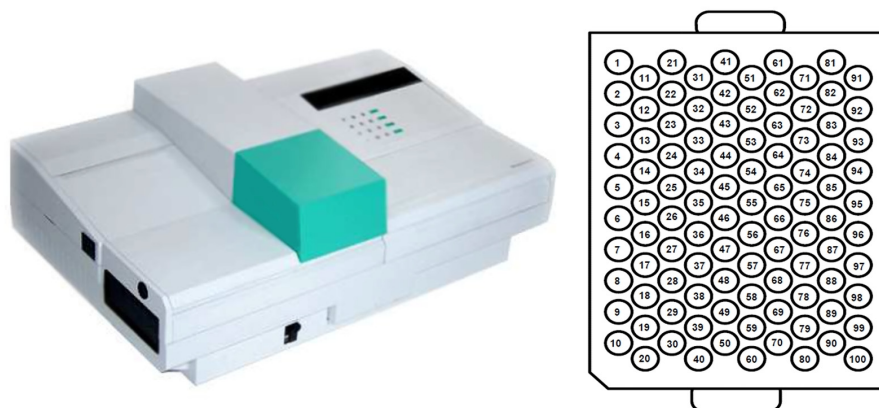


Fig. 2. Bioscreen microbiology reader on the left and honeycomb microplate on the right [4]

The testing sample contained 243 µL of nutrient broth, 27 µL of different soap concentrations and 30 µL of culture suspension. Soap controls contained 273 µL of nutrient broth and 27 µL of soap solutions were used as a blank for testing samples. The screening was done during 7 hours, with measurement on each hour, and shaking of microplates for 5 seconds before each measurement. Measurement of optical density in the wells was done at a wavelength of 610 nm. Each Bioscreen well was done in triplicate. The plots were built by software package Origin7.0 (Northampton, USA).

RESULTS AND DISCUSSION

Results obtained in agar dilution method are presented in Fig. 3. From the graph IC_{50} and IC_{90} values can be read. The IC_{50} value of the tested soap was 0.08 mg/mL while the MIC was detected at 0.41 mg/mL. Values above 0.75 mg/mL were found to be totally bactericidal (MBC) with no visible growth of colonies of *S. aureus*. When comparing these results to the other similar studies, the results obtained for our soap were evidently better than the ones from the study of soap activity which contained hexane extract of leaves of *Morinda morindoide*. The values obtained there for IC_{50} and MIC were 2.64 mg/mL and 31.25 mg/mL respectively against *S. aureus* by using agar dilution method [1]. The study which was using disk diffusion method for the determination of the antibacterial activity of medicated soap “Crusader” also had much higher values for MIC and MBC on *S. aureus* then in this study and these were: 62.5 mg/mL and 125 mg/mL respectively [16].

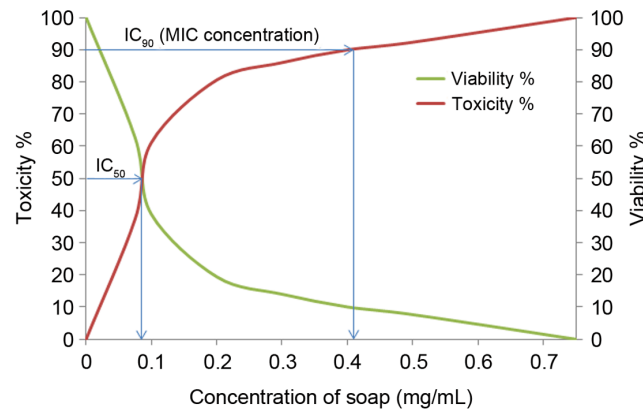


Fig. 3. Toxicity of different concentrations of soap solution and viability of the *S. aureus* SA812

The resulting absorbance for samples with added soap and controls and the percentage of inhibition in each measured point is presented in Table 1 and Table 2.

Table 1. Absorbance for the sample with added soap made from fresh oil and control, and inhibition percentage during the first 7 hours of experiment

Time (h)	Absorbance		
	Culture with added soap	Control	Inhibition (%)
0	0.0457	0.0457	0
1	0.1237	0.0767	0
2	0.1517	0.1327	0
3	0.1627	0.1690	3.7
4	0.1693	0.1887	10.2
5	0.1717	0.2190	21.6
6	0.1747	0.2530	31.0
7	0.1833	0.2907	36.9

Table 2. Absorbance for the sample with added soap made from fried oil and control, and inhibition percentage during the first 7 hours of experiment

Time (h)	Absorbance		
	Culture with added soap	Control	Inhibition (%)
0	0.0457	0.0457	0
1	0.1510	0.0767	0
2	0.1680	0.1327	0
3	0.1683	0.1690	0.4
4	0.1687	0.1887	10.6
5	0.1663	0.2190	24.0
6	0.1640	0.2530	35.2
7	0.1617	0.2907	44.4

Figure 4B and Fig. 5B shows the trends of growth for the sample with added soap and control. From slope coefficient we can read percentage of inhibition for soap sample against the control.

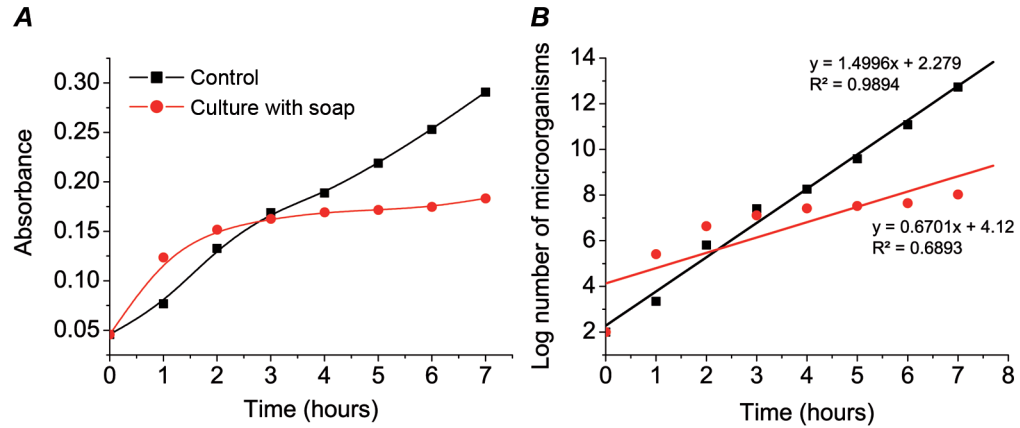


Fig. 4. Comparative graphs for growth of controls and culture with added soap made from fresh oil, A: absorbance change over time, B: logarithmic number of microorganisms change over time

The concentrations of 0.3 mg/mL of soaps made from fresh and from fried oils were taken for the experiments on Bioscreen microbiology reader. The optical density was measured during the first 7 hours of incubation in wells. On Fig. 4A and Fig. 5A can be seen that during the first two hours the solution with added soap showed higher absorbance in comparison to the control, but during the next 5 hours, it started to show a trend with lower slope coefficient.

The constant of growth for control was 1.4996, for culture with added soap made of fresh oil 0.6701 (Fig.4B), and for culture with added soap made of fried oil 0.4544 (Fig. 5B)

from which it can be calculated that overall inhibition of the growth of microorganism *S. aureus* during the first 7 hours. Inhibition was 55.3% when it was subjected to the soap made of fresh oil and 69.7% when it was subjected to the soap made of fried oil against the control. The obtained linear regression R^2 was acceptable for the growth of bacteria colonies with the addition of soap made of fresh oil and it was 0.6893 (Fig. 4B). On the other hand, growth trend line of bacteria subjected to soap made from fried oil was obtained with regression 0.3627 (Fig. 5B), which cannot be accepted as valid for accurate assessment of the results. The possible reason might be in the optical interference of some compounds which might be present in soap made of frying oil, and which could have occurred during the screening process.

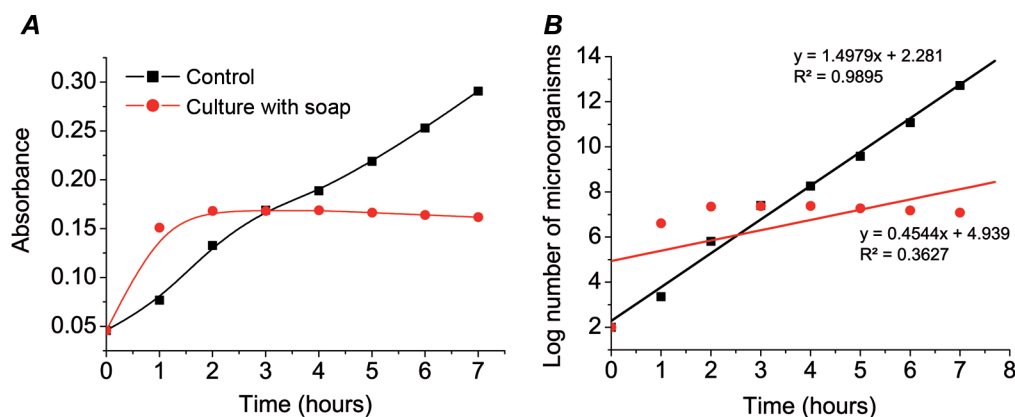


Fig. 5. Comparative graphs for growth of controls and culture with added soap made from fried oil, **A**: absorbance change over time, **B**: logarithmic number of microorganisms change over time

The two approaches were used: the agar dilution method for determining the MIC and automatic Bioscreen system for the following kinetics. The agar dilution method gave us some concrete values as a result but it required a lot of work in preparation of the single experiment. In order to compare it with other methods, it's necessary to standardize the number of microorganisms treated. It was proven that antibiotics were more potent while affect the lower number of colonies in the experiments and vice versa [11]. Thus the Bioscreen came up as a solution as it has many options with the possibility of screening of several samples in different concentrations including the controls. The obtained results are then easily and accurately comparable [22]. 24 hours of work on Bioscreen can replace more than 30 days of manual work in the laboratory, which attribute the Bioscreen as a very powerful device for in vitro microbial analysis [4].

Finding the best methodology is important for further development and supporting the production of natural soaps and their assessment. The advantage of natural soaps over commercially available ones is that they do not contain chemicals which are in a lot of cases added in order to increase the antibacterial activity. The triclosan is very often the ingredient of liquid soaps and it represents one of the biggest pharmaceutical contaminant found in river samples in the USA. Triclocarban is mostly present in bar soaps. Those chemicals can also come to the human food chain since triclosan was detected in human breast milk [2]. These findings are emphasizing the importance of constant search of natural sources for antimicrobial agents, both for nature and human population.

CONCLUSIONS

The study emphasized the issues concerning the antimicrobial determination of natural produced soaps. The differences observed between soap samples produced from heat-treated and not heat treated edible plant oils are also indicating the specific characteristics of these two products. The soaps produced from heat-treated (fried) oil probably will be more present on the market, since this raw material for the soap production represents an ecological solution for the waste material, such as used oil from the food industry and restaurants. The main characteristic of soap is its antimicrobial properties and in our study, we tested these properties with two methods (traditional cultivation and Bioscreen methodology). Bioscreen methodology is less time consuming than cultivation method and also due to less human manipulation, there is a lower chance of technical mistakes. The research showed significant differences between results gained by Bioscreen methodology between soaps produced with heated and not heated oil. These results are pointing out the necessity of different sample preparation for Bioscreen. The impurities in soaps produced from used oil can be the possible reason for these observed differences. Though, this hypothesis will be tested in our ongoing and future experiments. The conducted research gained important information about antimicrobial analysis of homemade soaps, especially because this kind of experiment, by our knowledge, has not been described yet.

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