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Germicidal Properties of Ozonated Sunflower Oil

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The aim of this work was to establish the minimal inhibition concentration (MIC) of ozonated sunflower oil for various microorganisms. To determine the influence of the ozonated medium on the growth of bacteria Bacillus subtilis, Escherichia coli and yeast Candida albicans the Petri dish method was used. Chemical and physical properties of ozonated sunflower oil were additionally studied. Microbiological studies proved that these microbes have various sensibility against ozonated oil. The most resistant are gram-negative bacteria E. coli and the yeast C. albicans. Gram-positive bacteria, B. subtilis turned out to be less resistant, because no growth was observed for preparation with an ozone dose of 200 mgO3/g oil.

Keywords Ozone, Ozonized Sunflower Oil, MIC, Disinfection, Germicidal Properties

INTRODUCTION

Ozone is a well-known allotropic form of oxygen and a very strong oxidative agent. Due to this property it is one of the strongest disinfectants. Its biocidal activity was discovered by Fox in 1873. Ozone was first used in medicine in 1894 by Labbe and Oudin for tuberculosis treatment. The 20th century brought many new applications for ozone germicidal properties. A gaseous mixture of ozone and air was used in 1915 for wound disinfection by Wolff. In 1935 it was used in dental medicine by Fisch and in surgery by Payr (Viebahn-Hänsler, 2002).

Ozone germicidal action was widely proved on a broad group of microorganisms including gram-positive and gram-negative bacteria as well as spores and vegetative cells (Guzel-Seydim et al., 2004). Many studies comparing ozone with popular disinfecting agents have been carried out (Khadré and Yousef, 2001; Telles Silveira et al., 2005; Filippi, 2005; Orta de Velasquez et al., 2005; John et al., 2005). The results of the studies suggest that ozone is the best disinfectant. Special attention is deserved by studies by Telles Silveira et al., who confronted germicidal action of ozone and sodium hypochlorite on Enterococcus sp. (gram-positive bacteria) which are often found in hospital sewage. Ozone turned out to be effective against these microorganisms, even the vancomycin-resistant Enterococcus (VRE), whereas sodium hypochlorite proved to be less effective (Telles Silveira et al., 2005).

Another significant study was conducted by Filippi, who investigated the survivability of the pathogens in dentistry equipment disinfected with ozone and hydrogen peroxide. Samples from devices disinfected with hydrogen peroxide showed the presence of Pseudomonas aeruginosa, whereas in samples from devices disinfected with ozone the occurrence of P. aeruginosa was not observed (Filippi, 2005). Another study comparing the biocidal action of ozone and hydrogen peroxide against foodborne Bacillus spp. spores was conducted by Khadré and Yousef. They proved that hydrogen peroxide is less effective than ozone even when it was used in concentrations 10,000-fold higher than those applied for ozone (Khadré and Yousef, 2001).

Ozone kills microbes by acting on cell walls and semi-permeable membranes. This leads to changes in the chemical structure of the cell and interferes with cell metabolism products exchanging with the environment (Biń, 2002; Shiota et al., 2005). Furthermore it causes cell envelope disintegration leading to leakage of the cell contents (Guzel-Seydim et al., 2004; Pascual et al., 2007). Bactericidal, fungicidal and virucidal properties of ozone are attributed to its ability to destroy many of the enzymatic structures. Naturally each microorganism has specific sensitivity to ozone. Bacteria are more sensitive than yeast and fungi. As a result of differences in structure of the cell walls, gram-positive bacteria are more sensitive to ozone than gram-negative organisms (Guzel-Seydim et al., 2004; Pascual et al., 2007).
Nowadays methods exploiting medical applications of ozone are called ozone therapy, which may be employed in different fields of medicine, e.g., general medicine, dermatology, surgery, dental medicine, orthopedics, immunology (Viebahn-Hänsler, 2002). Since ozone is a gas and there are not many medicines available in gaseous phase, special techniques had to be developed for the safe use of ozone (Viebahn-Hänsler, 2002). It can be administered in the form of ozone-oxygen mixtures, ozonized water or ozonized vegetable oils. Although gaseous ozone is used in medicine for treating patients, that does not mean that ozone is harmless. When breathing ozone in at high concentrations it can be a very harmful substance, especially for the pulmonary tract. Hence it is extremely important to use ozone in small and safe doses just above the threshold level (Bocci, 2006).

As Bocci informs, the U.S. Clean Air Act recently has established a new ozone level of 0.06 ppm (120 μg/m³) as an 8-hour mean concentration. That is why ozonated water and vegetable oils are preferable. Each of them is used for different ailments, e.g., ozone-oxygen mixtures are applied mainly for flesh-wounds like diabetes foot. Whereas ozonized water is used rather on fresh, infected wounds as well as against mycosis, it is very popular in dentistry for disinfection and treatment of wounds after tooth extraction. According to Shiotya et al., it is also used for the disinfection of the eye before an operation and in ophthalmology (Shiotya et al., 2005).

Vegetable oils have found application in wound disinfection and improve the process of healing. They are interesting because they can be used outside a surgery. Their disinfecting effect is much slower than in the case of ozone-oxygen mixtures or even ozonated water. Ozonated oils need hours rather than seconds to achieve a satisfactory reduction in the amount of microorganisms (Viebahn-Hänsler, 2002). Ozonated oils are quite popular in many countries especially in Cuba where OLEOZONE is produced according to the process developed at the Ozone Research Center. It is a registered drug in Cuba and is obtained through the ozonation of sunflower oil. There are many other commercially produced ozonated vegetable oils on the market like Cocozone – made from coconut oil in Great Britain, OOO (ozonized olive oil) – made in Canada, O2-Zap – made from olive oil in USA (available from websites: www. ozoneservices.com/products/000; www.glchealth.com/ozonated-olive-oil-ozone.php; www.cocozone.co.uk).

New methods of pathogenic microorganism elimination have aroused much interest recently; that is why ozone and its application as a disinfection agent has become a popular object of scientific research. The aim of this study was to determine minimum ozone doses in ozonated oil for killing various microorganisms like Bacillus subtilis, Escherichia coli, Candida albicans. First, five different vegetable oils were ozonated and their properties, such as iodine number, peroxide value, acidity value and viscosity were compared. Afterwards one chosen vegetable oil was subjected to further microbiological studies.

### MATERIALS AND METHODS

Pure oil (150 cm³) was introduced into a bubble column reactor at room temperature. The oxygen-ozone mixture with ozone concentration around 30 mg/dm³ was bubbled through the given oil with the volumetric flow of 20 dm³/h. The reaction was conducted for 1 up to 50 hours, respectively, to the desired ozone dose (max. 300 mg O₃/g oil).

The experimental setup is shown in Figure 1. Oxygen was obtained from air by the PSA (Pressure Swing Adsorption) oxygen generator AirSep, Buffalo, New York (1). The gas flow rate was adjusted with a rotameter (2). After dehumidification in a drying jar (3) the gas was fed into the Ozone Generator BMT 802X, Berlin, Germany (4). Next, the oxygen-ozone mixture passes through the Ozone Analyzer BMT 963 (5), which measures ozone concentration in the gas at the reactor inlet. The reaction between ozone and a vegetable oil takes place in the reactor (6). After leaving the reaction space the gas reaches another Ozone Analyzer BMT 964 (7), which measures the outlet ozone concentration.

Peroxide index, acidity index, iodine number and viscosity were used for determining the quality of ozonized sunflower oil. All chemical analysis were carried out according to ISO Standards: Iodine number – PN-ISO 3961:1998, peroxide index – PN-ISO 3660:1996, acidity index – PN-ISO 660:1998, whereas viscosity was estimated with the use of a rheometer (Bohlin Instruments CVO 120 High Resolution, Malvern, UK).

### MICROBIOLOGICAL STUDIES

Ozonized sunflower oils with ozone doses around 20, 40, 70, 100, 200, 250 and 300 mg O₃/g oil were used in microbiological studies. The bactericidal and fungicidal effectiveness of these specimens was investigated. The research was conducted with the use of the Koch’s pour plate method to estimate the influence of ozonized...
sunflower oils on gram-positive bacteria *Bacillus subtilis*, gram-negative bacteria *Escherichia coli*, and yeast *Candida albicans* (from clean culture collection of the Industrial Microbiology and Biotechnology Department of the Technical University of Łódź). The strains of yeast were cultured on YPG medium, whereas bacteria were cultured on enriched agar as a medium. In order to assess the influence of ozonated sunflower oil on microbial growth, the ozonated sunflower oils and pure sunflower oil as control were added to inoculated plates and then cultivated. Applying ozonated oil with various doses we looked for case where no growth was observed.

RESULTS AND DISCUSSION

Physical and Chemical Properties

The first stage of this research pursued a goal of assessing the changes in chemical properties of ozonated vegetable oils. Five vegetable oils with different compositions of fatty acids were tested: olive oil, sunflower oil, rapeseed oil, corn oil and grape seed oil (Table 1). All five oils were ozonated until reaching the absorbed ozone dose around 10 mg O₃/g oil. Results were gathered in Table 2. The highest initial iodine number was observed for sunflower oil, the lowest value was found in olive oil. This is connected with the high amount of unsaturated, especially polyunsaturated, fatty acid in sunflower oil and their low amount in olive oil. For all vegetable oils examined, a decrease in iodine number after ozonation was observed, obviously as a result of reaction between ozone and unsaturated fatty acids.

It varied depending on the tested oil, which again is related to the composition of oils. Another analyzed parameter was peroxide index which increased during the ozonation process. The highest values were obtained for ozonated olive oil and ozonated sunflower oil. High peroxide index is connected with the reaction of ozone with carbon-carbon double bonds in unsaturated fatty acids. It produces ozonides, hydroperoxides, aldehydes, peroxides, diperoxides and polyperoxides according to well-known Criegee mechanism (Diaz et al., 2005; Jardines et al., 2003). Furthermore according to Tamoto et al. the major component of ozonated olive oil is triozonide of triolein (Figure 2). The increase of peroxide index for sunflower oil was higher than for olive oil due to lower amounts of double bonds in olive oil. Our studies found confirmation in studies performed by Diaz et al. (2005a, 2005b, 2006). Taking into account the price of these two oils, sunflower oil turns out to be more favorable for this particular process. Therefore further studies were performed only on sunflower oil. Results were gathered in Table 3.

Iodine number analysis for ozonized sunflower oil showed a decrease of iodine number with an increasing ozone dose. The decrease in iodine number for the examined range of the ozone dose was linear as shown in Figure 3. The iodine number for ozonized medium with low ozone doses: 4.05, 20.27, 27.81 (mg O₃/g oil) dropped only by 1.1%, 6.6% and 10.3%, respectively. With the use of elongated reaction time, thereby higher ozone dose, the decrease of iodine number was rising and for 300 mg O₃/g oil it reached 97.1%. This shows that almost all double bonds present in fatty acids were saturated in the reaction with such a strong oxidative agent as ozone.

From the germicidal point of view, the most important is peroxide number since it indicates the amount of peroxide compounds formed. As Diaz et al. suggested, these are responsible for the biocidal activity of ozonized vegetable oils (Diaz et al., 2005a, 2005b, 2006). Peroxide number increases almost linearly with the increase of

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<th>TABLE 1. Comparison of Fatty Acids Composition in Studied Vegetable Oils</th>
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<td>Oil</td>
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<td>Olive oil</td>
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<td>Rapeseed oil</td>
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<td>Grapeseed oil</td>
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<td>Sunflower oil</td>
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<tr>
<td>Corn oil</td>
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<td>Manufacturer (country)</td>
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<tr>
<td>Costa D’Oro (Italy)</td>
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<td>ZT “Kruszwica” S.A. (Poland)</td>
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<td>Monini (USA)</td>
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<td>SALVADORI Costanza (Italy)</td>
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<th>TABLE 2. Chemical Parameters for Five Ozonated Vegetable Oils</th>
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<td>Oil</td>
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ozone dosage as shown in Figure 4. Peroxide number for the ozone dosage 40 mgO₃/g oil rose forty times compared to pure sunflower oil, whereas for 300 mgO₃/g oil it rose more than 100 times.

Chemical analyses of ozonized sunflower oil proved that acidity number increases with increase of ozone dosage. Even for short ozonation times, the acidity number increased more than 30 times. This relation is presented in Figure 5. Conventionally, acidity number is used to assess if the studied oil is fresh or rancid, and if it was well stored. The growth in the value of the acidity number indicates the correctness of the ozonation process. Fats show neutral reaction, however partial hydrolysis takes place during the ozonation process and unbound fatty acids give fats an acidic reaction. Apart from chemical and biological properties, viscosity also was analyzed. Results showed increases in viscosity of ozonated sunflower oil with increasing ozone dose. This phenomenon could be used for process monitoring in order to estimate ozonation reaction progression. The two ozonated sunflower oils with the highest ozone doses had the consistency of gel (viscosity achieved app. 0.28 Pas).

Microbiological Studies

Microbiological studies for ozonated sunflower oil were carried out with the following ozone doses: 20, 40, 70, 100, 200, 250 and 300 mg O₃/g oil. Bactericidal and
fungicidal effectiveness of ozonized sunflower oil was investigated.

The examination of the results obtained allowed determination of the ozone dose in preparation that causes 100% growth inhibition of the examined microorganisms. Results were gathered in Table 4.

The findings suggest that microbes have diversified sensitivity to ozonated sunflower oil. Gram-positive bacteria *B. subtilis* turned out to be the least resistant, because no growth on the plates for preparation with the ozone dose of 200 mg O₃/g oil and above was observed. Both gram-negative bacteria *E. coli* and yeast *C. albicans* were more resistant to ozonated sunflower oil than *B. subtilis*, since these germs needed preparation with the ozone dose higher by 50 mg O₃/g oil to be totally destroyed. This could suggest that ozonated sunflower oil, similarly to gaseous ozone, acts stronger on gram-positive bacteria than it does on gram-negative bacteria and fungi. As explained in the introduction, this is connected with the cell wall structure. However, after conducting a further literature survey, it is obvious that such an approach would be too simplified. Since the Diaz et al. studies on antimicrobial action of ozonated vegetable oils, not all gram-positive bacteria are more sensitive to ozonated oils than gram-negative ones. This suggests that the mechanism of biocidal action of ozonated oils may be more complex.

Exemplary fungicidal effects of ozonated sunflower oil is shown in Figure 6. Ozonized sunflower oil with the ozone dose below 200 mg O₃/g oil shows no germicidal properties. Then, 100% growth inhibition of the studied microorganisms was obtained for ozonated sunflower oils with high values of peroxide number (more than 630 milliequivalent/kg). For ozone doses lower than 200 mg O₃/g oil and the peroxide number lower than 630 milliequivalent/kg, no disinfecting effect was gained. Our studies confirmed the results of the earlier research by Diaz et al. They suggested that there exists a connection between the value of the peroxide number and the disinfecting properties of ozonated sunflower oils. The results indicated that ozonated sunflower oils can inhibit the development and growth of the microorganisms tested.

*Therapeutic Effects*

Additionally, therapeutic effects of ozonized sunflower oil with ozone doses around 300 mg O₃/g oil were tested with the cooperation of the Dermatology Clinic of the Medical University of Łódź. The preparation was used for one month for topical treatment of two patients, one with a wound ulcer and the other with nail psoriasis. In both cases positive results were obtained as shown in Figure 7. In Figure 7A, the wound ulcer before treatment can be observed, with a high amount of fibrous tissue and only few areas with granular tissue, which is the sign of healing. After a month of therapy (Figure 7B) a significant increase in amount of granulation is observed. In the case of nail psoriasis, presented in Figure 7C and 7D, the improvement of patient condition is easily noticeable.

*CONCLUSIONS*

Among the studied microorganisms, the most resistant against ozonated oil appeared to be gram-negative bacteria *E. coli* and yeast *C. albicans*, which required around 250 mg O₃/g oil to become inhibited. Whereas gram-positive bacteria *B. subtilis* was shown to be the least resistant because no growth on the plates for preparation with the ozone dose 200 mg O₃/g oil was observed. Our studies confirmed the results of the earlier research by Diaz et al. They suggested that there exists a connection between the value of the peroxide number and the disinfecting properties of ozonated sunflower oils. The results indicated that ozonated sunflower oils can inhibit the development and growth of the microorganisms tested.

Ozonated sunflower oils gain recognition since they have one significant advantage over gaseous ozone and ozonized water. It can be used outside a surgery by the patient himself. It is also easy to manufacture and to be preserved. Furthermore, its gelled consistency makes it easy to apply locally. In order to provide the required effectiveness of topical therapy for scarcely healing wounds, it is important to apply ozonated oil with the ozone dose exceeding the minimal dose for the specific pathogen.
ACKNOWLEDGMENTS

The authors thank Prof. Elżbieta Waszczykowska for conducting the clinical studies on the ozonated sunflower oil.

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19. <www.cocozone.co.uk/>. .

FIGURE 7. A – the before photo of the wound ulcer, B – the photo of the wound after one month treatment, C – the before photo of the nail psoriasis, D – the photo of the nails after one month of treatment (Dermatology Clinic of the Medical University of Lodz).