DETERMINING THE SUITABILITY OF PHOTOSENSITIZERS AS BIOCIDES IN THE SOAKING PROCESS

by

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ABSTRACT

All photosensitizers kill the microorganisms in the environment by generating singlet oxygen with the energy they receive from sunlight. Oxygen has a toxic effect not only on anaerobic types, but also on the living beings that depend on oxygen to survive. This study aims to explore the neutralization or the restriction of the effects of the bacteria in the float during the soaking process by using the oxygen effect of chlorophyll, which is a photosensitizer that all green plants in nature contain in great amounts. For this purpose, the chlorophyll extracted from spinach leaves was used in the soaking process and its effect on the total aerobic mezophyll bacteria was determined in comparison with the effect of bactericides.

ABSTRACTO

Todo fotosensitivizante mata microorganismos en el medio ambiente generando oxígeno activado por medio de la energía recibida de la luz solar. Tal oxígeno tiene un efecto tóxico no solo sobre anaerobios, sino sobre seres vivientes que dependen del oxígeno para sobrevivir. Este estudio ambiciona explorar la neutralización o inhibición de los efectos de las bacterias en el baño del proceso de remojo utilizando el efecto oxigenante de la clorofila, que es un fotosensitivizante natural que las plantas verdes contienen en grandes cantidades. Para este propósito, clorofila extraída de hojas de espinaca se utilizó en el proceso de remojo y su efecto sobre la totalidad de bacteria mezofílica aeróbica fue determinado comparativamente con otros bactericidas.

INTRODUCTION

A Photosensitizer is a molecule that absorbs energy in the visible spectrum of light and transfers this energy into substrata or molecular oxygen. Primary molecules that can be used as photosensitizers include methylene blue, rose bengal, eosin, toluidin blue, chlorophyll, hemetaporfirin, hemato-porphyrin and several heterocyclic compounds.[1] All photosensitizers generate singlet oxygen with the energy they get from sunlight, and they kill the microorganisms in the environment. Di-radicalic oxygen with high reactivity is called singlet oxygen.[1,2] It can again turn into oxygen after giving its energy to the environment in the form of wave energy. Oxygen has a toxic effect not only on anaerobic types, but also on living beings that definitely depend on oxygen in order to survive. The toxic effect of oxygen occurs in two ways, namely the inhibition of some enzymes by molecular oxygen, or the creation of poisonous effects by oxygen radicals. The energy of the first type sigma singlet oxygen is more, yet it is short-lived. The second type delta singlet oxygen is longer-lived and accepted to be the form fundamentally responsible from the observed chemical reaction.[3] These two reactive oxygen types[4] are recognized to be biologically effective in producing microorganism deaths. Photosensitizers harm the bacteria with the second type mechanism of photooxidation, and these molecules are introduced as potential alternatives to known antibiotics.[5] Chlorophyll, which is a photosensitizer, is the green pigment responsible for photosynthesis. Chlorophyll, which gives the plants their green color, is a photoreceptor that exists in the chloroplast of green plants. The basic structure of chlorophyll molecule is formed by the porphyry ring attached to a central atom and a long carbon chain (phytol).[6] (See Figue 1) Antibacterial effect of porphyry reveals distinctive characteristics in photodynamic treatment.[7] The structure of the chlorophyll is identical with the hemo structure in hemoglobine. The only difference is that the central atom of hemo structure is iron.[8] Chlorophyll turns the energy it gets from sunlight into oxygen and sugar. All green plants contain high amounts of chlorophyll in their structure; for example, spinach contains 1% chlorophyll at its dry weight.[8,9] This study has aimed to explore the neutralization or the restriction of the effects of the bacteria in the float during the soaking process by using the singlet oxygen effect of chlorophyll. To this end, chlorophyll was used in soaking process.

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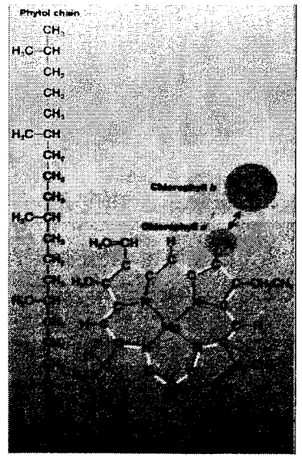

Figure 1. - Chlorophyll structure

Raw hide bears millions of bacteria that come from the soil and fertilizers, and survive in skin flora even when the animal is alive.[10,11] The activity of these bacteria is restricted by conservation. Through the soaking process that constitutes the first process in the modification of raw skin into leather; the bacteriatoxic effect of conservation salt on the skin disappears while some of the globular protein on the skin and the filth such as salt, blood and urine are removed, and the skin structure regains the water it has lost during conservation. In the meantime, the microorganisms in the environment are reactivated and the skin is exposed to microorganism attacks.[11] Various germ spores, which survive during conservation and drying, multiply again in soaking floats. The microorganism activity observed in raw skins during soaking process causes serious damages in leather quality. In prolonged soaking floats, defects occur in the raw hide such as irredeemable hair slip and hair shed, matte and lusterless grain, grain crack, grain break and damage in epidermis layer.[12,13] The use of biocides is suggested so as to prevent these damages.[14,15]. When biocide is not used, millions of bacteria proliferate in 4 to 6 hours in every millimeter of soaking liquor depending on pH, temperature and the filth load on the hide.[16] As the soaking process period reaches to 24 hours, possible damages on the hide increase depending on the amount of bacteria. Microorganism activities can be restricted by means of using biocide in the soaking float.[16,17] However, these substances that are toxic for microorganisms are also toxic for humans and other living beings. Several biocides are known to have harmful effects on the environment. When released to environment, they cause serious ecologic effects on water, soil and food chain. For example, they destroy the primer productivity of water by remaining in water for a long time without decomposing; they have negative effects on zooplankton eggs, larva and adults; they may cause acute intoxication in human beings through respiratory, skin and oral intake, and may result in chronic intoxication when taken trough consumed plants infected by biocides.[18]

Due to increasing environmental pressures today, the use of substances harmless to environment and human health are highly emphasized in every step of leather production. Accordingly, this study that we have carried out for this purpose explores the usability of chlorophyll as a photosensitizer in order to prevent the microorganism activities during soaking process.

EXPERIMENTAL

Materials

Raw Hide

7 salted-dry, native brand sheep raw hide was used in the research. 4 pieces weighing 100 grams were cut in aseptic conditions from the back part of each raw hide. The study was repeated 7 times with 28 hide samples in total.

Chlorophyll

Chlorophyll exists in nature in many plants and algae to a great extent. In this study, chlorophyll was extracted from fresh spinach leaves (*Chenopodiaceae*). The reason for preferring spinach is the fact that it includes high amounts of chlorophyll, it can be obtained easily and it is cheap.

Light Source

The study on the group soaked for 8 hours was carried out under day light. Sunlight was used during daytime, and 230-240 W, 50-60 H Philips ecotone bulbs that give white day light was used at night in the group treated with 24 hours soaking process.

Oxygen Source

Air compressor was used as oxygen source in the soaking float of hides treated with chlorophyll.

Bactericide

Bactericides that are widely employed in trade were used in the experiment for the purpose of comparison. The utilized bactericides were obtained as unopened packing from a bactericide manufacturer. Commercial bactericides that are widely used in leather industry for this purpose and contain 7-25% phenol, 4-chloro-3 methyl, sodium were used in this study.

Nutrient Media

PCA (Plate Count Agar) (Oxoid) nutrient media was used in the examination for the total aerobic mezophyll bacteria count.

Pepton Water

0.1% peptone water was used in the dilutions before the total aerobic mezophyll bacteria count.

Methods

Chlorophyll Extraction

Chlorophyll can be obtained from all green plants. The chlorophyll used in this study was extracted from spinach

leaves. The extraction process and chlorophyll determination were done in accordance with the method indicated by AOAC in 1984. After removing the water and acetone from its content, the maximum absorbance values of the chlorophyll extract were measured with a Shimadzu UV-1601 spectophometer at 645 nm and 660 nm.[20] Then, the amounts of total chlorophyll, chlorophyll-a and chlorophyll-b in 1 g of spinach leaf tissue were calculated as mg/g according to the formulae below:

Chlorophyll-a = $9.93 \times A_{660} - 0.777 \times A_{645}$

Chlorophyll-b = $17.6 \times A_{660} - 2.81 \times A_{645}$

Total chloropyll = $7.12 \times A_{660} + 16.8 \times A_{645}$

The approximate values determined on the spinach leaf samples used in this study are as follows:

Chlorophyll-a = 0.59 mg/g
Chlorophyll-b = 0.34 mg/g
Total chlorophyll = 0.93 mg/g

Soaking Process

Soaking process was applied under laboratory conditions in accordance with traditional methods. The mechanical affect needed in the soaking process was provided by a shaker applied for 10 minutes out of every hour. The wetting of salt-dry hides was realized in two steps. In the first step, 2 hours pre-soaking process was applied without adding any preserving agent. The aim of this comparatively short process was to let the hides take in some water and to remove the substances like dirt, blood, conservation salt, dust and dung from the hide. In the second step, which constitutes the main soaking process, bactericide and chlorophyll were added to each float.[21,22,23]

TABLE I
General Formulation for the Soaking Processes with Bactericide (8h)

Process	%	Product	Temperature (°C)	Time (h)	pH
Pre-soaking	1000	Su	27	2	7
Soaking	1000	Su	27		
	1	Bactericide		8	7

TABLE II
General Formulation for the Soaking Processes with Chlorophyll (8h)

Process	%	Product	Temperature (°C)	Time (h)	pH
Pre-soaking	1000	water	27	2	7
Soaking	1000	water	27		
	0.5	chlorophyll		8	7

TABLE III
General Formulation for the Soaking Processes with Chlorophyll (8h)

Process	%	Product	Temperature (°C)	Time (h)	pH
Pre-soaking	1000	water	27	2	7
Soaking	1000	water	27		
	1	chlorophyll		8	7

TABLE IV
General Formulation for the Soaking Processes (8h)

Process	%	Product	Temperature (°C)	Time (h)	pH
Pre-soaking	1000	water	27	2	7
Soaking	1000	water	27	8	7

TABLE V
General Formulation for the Soaking Processes with Bactericide (24h)

Process	%	Product	Temperature (°C)	Time (h)	pH
Pre-soaking	1000	Su	27	2	7
Soaking	1000	Su	27		
	1	Bactericide		24	7

TABLE VI
General Formulation for the Soaking Processes with Chlorophyll (24h)

Process	%	Product	Temperature (°C)	Time (h)	pH
Pre-soaking	1000	water	28	2	7
Soaking	1000	water	28		
	0.5	chlorophyll		24	7

TABLE VII
General Formulation for the Soaking Processes with Chlorophyll (24h)

Process	%	Product	Temperature (°C)	Time (h)	pH
Pre-soaking	1000	water	28	2	7
Soaking	1000	water	28		
	1	chlorophyll		24	7

TABLE VIII
General Formulation for the Soaking Processes with Chlorophyll (24h)

Process	%	Product	Temperature (°C)	Time (h)	pH
Pre-soaking	1000	water	28	2	7
Soaking	1000	water	28	24	7

The hides were classified into four groups in the experiment. The first group of hides was named as control group, and after 2h pre- soaking process they were treated with two separate soaking processes of 8h and 24h without adding any preserving agent. 2h pre-soaking process was applied also to the second group, and 1% bactericide as suggested in the prospectus of the manufacturer was added to the float before 8h and 24h soaking process. After 2h pre-soaking process, 0.5% and 1% chlorophyll on raw hide weight was added to the main soaking float in the third and fourth group of hides respectively. As in other groups, 8h and 24h soaking process was applied to these groups. During and after the soaking process, bacteria growth was determined and the microorganisms in the float were counted by taking samples from the float at the end of 8h and 24 hours. The formulations shown in Tables I - VIII were used in this study and the hides were processed into leather after the soaking process by the same formulation.

Microbial Count Method

For total aerobic bacteria and spore count, a 1 ml water sample was taken from soaking float of the control group and from the soaking floats containing bactericide and chlorophyll. These samples were transferred to tubes that contained 9 ml dilution sediment, and samples were diluted from 10^1 to 10^6. Diluted samples were implanted into culture environments by pour plate method. The petri dishes were incubated for 48 hours at 37 °C for total aerobic bacteria count. All implantations within the scope of this study were duplicated, and count results were obtained by arithmetic average calculation at the end of incubation periods from petri dishes with 30-300 colonies. The total number of living microorganisms in the soaking liquor was calculated from the number of colonies formed in petri plates at the end of 48 hours, and count results were given as colony forming units per milliliter (cfu/ml).[24,19]

Results and Discussion

Raw hide is composed of 64% water, 30% protein, 2.5-3.5% carbohydrate, 2-2.5% fat and 0.3-0.5% mineral.[25,26] Raw hide compounds form a highly comfortable environment for microbial growth. Especially various types of proteolytic bacteria genus, such as *Bacillus, Proteus, Pseudomanas, Clostridium and Lactobacillus* were isolated from soaking liquors and were defined.[27,16] During the soaking process, which constitutes the initial process in the modification of raw hide into leather, the hide regains the water it has lost in conservation while some of the globular proteins and the dirt like salt, blood and urine on the hide are removed. The hide is exposed to microorganism activities as the conservation effect on the raw hide disappears through this process. Since soaking floats form the necessary living conditions for bacterial growth, it is possible to observe millions of bacteria in every milliliter of the soaking float in 4-6 hours.[16] Sari has stated that 1 gram of salted-wet sheep hide contains 2.0×10^8 microorganisms, 95% of which exist on the wool. Some researchers acknowledge that the number of bacteria in soaking float would cause problems when it exceeds 10^5 cfu/ml, and it would be ideal if the number is below this value.[28] There are more than 80

TABLE IX
Experiment Results Obtained at the End of 8 and 24 Hour Soaking Periods

Product	Colony Number (cfu/ml) (8h) (Average)	Colony Number (cfu/ml) (24h) (o Average)
Bactericide (1%)	$1.68x10^4$	$2.25x10^6$
Chlorophyll (0.5%)	$1.05 x10^4$	$1.65 x10^6$
Chlorophyll (1%)	$6.30 x10^3$	$7.55 x10^5$
Control	$1.74 x10^4$	$2.72 x10^6$

aerobic and anaerobic microorganism types that spoil and putrefy hide, and 70% of these microorganisms are known to be aerobic while the rest is anaerobic.[27]

It has been mentioned in various studies that singlet oxygen and superoxide radicals are effective in causing microorganism deaths in photodynamic processes.[29,4,5] In this study, which we have carried out for the purpose of examining the effect of chlorophyll as a photosensitizer on the bacteria in soaking float, 2 hours pre-soaking process was followed by 8h and 24 hours soaking process. 1% bactericide, or 0.5% or 1% chlorophyll was added to each soaking float, and the number of aerobic mezophyll bacteria grew in water at the end of 8h and 24 hours was determined by microbiologic count methods. The number of total bacteria colonies in soaking float was determined to be $1.05x10^4$ cfu/ml at the end of 8 hours soaking process applied by adding 0.5% chlorophyll to the new float following 2 hours pre-soaking process, while the number of total aerobic bacteria in float was found to be $6.30x10^3$ cfu/ml at the end of 8 hours soaking process with 1% chlorophyll (Table IX). The number of total aerobic bacteria was determined to be $1.68x10^4$ cfu/ml in the samples taken from 8 hours soaking float where 1% bactericide was used; on the other hand, the number of total aerobic bacteria in the soaking float from the control group was observed to be $1.74x10^4$ cfu/ml (Table IX).

Yapici, *et. al.* have determined that the total number of aerobic mezophyll bacteria in the float as $1.5x10^8$ cfu/ml at the end of 4 hours soaking process without bactericide. Whereas, the number of total aerobic mezophyll bacteria in the new float has been found to be $8.0x10^6$ cfu/ml at the end of 24 hours period of soaking process without bactericide.[21]

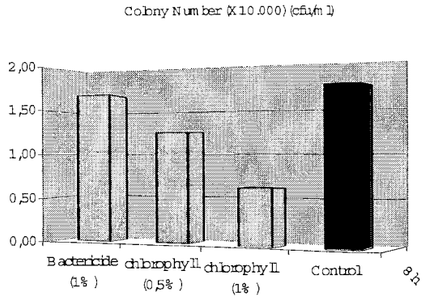

Figure 2. - The total number of aerobic bacteria at the end of 8 hours soaking

When the research results were examined, the number of bacteria was determined to be $1.74x10^4$ cfu/ml in the control group obtained from the same hide and treated with 8 hours soaking process; whereas $1.05x10^4$ cfu/ml bacteria was found in the soaking float with 0.5% chlorophyll at the end of 8 hours period. The growth of total aerobic bacteria is restricted by adding 0.5% or 1% chlorophyll to the soaking float. However, it was observed that the number of microorganisms in the float with 1% chlorophyll was less; in other words, the number of bacteria colonies in the soaking float was observed to decrease as the amount of chlorophyll increased.

Table IX reveals that the total number of aerobic bacteria colony in soaking float with 0.5% chlorophyll is $1.65x10^6$ cfu/ml at the end of 24 hours period, while this number is found to be $7.55x10^5$ cfu/ml in the process with 1% chlorophyll. The total number of aerobic bacteria was determined to be $2.25x10^6$ cfu/ml after the microbiologic counts of samples taken from soaking floats with bactericide, while this number was detected as $2.72x10^6$ cfu/ml at the counts of the control group (Table IX).

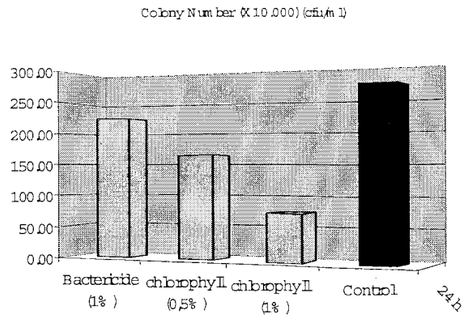

Figure 3. - The total number of aerobic bacteria at the end of 24 hours soaking

It was observed in the study that the color of soaking float with chlorophyll, which was green in the beginning, turned into yellow toward to end of the process. This transformation occurred in a shorter period in the process with 0.5% chlorophyll, while it lasted longer in the process with 1% chlorophyll. The color of the float is thought to change with the decrease in the effect of chlorophyll as a result of the photochemical reactions of chlorophyll with the microorganisms in the soaking float.

CONCLUSIONS

When the data obtained from this research are examined, it can be concluded that the bactericide and chlorophyll added in soaking float were effective against bacteria in both proportions. Yet, 8h and 24 hours processes with 0.5% and 1% chlorophyll were observed to be more effective on bacteria. This effect is increased with increasing amounts of chlorophyll. According to the data, chlorophyll, which is easy to obtain from every green plant in nature, can be used during the soaking process as an alternative and environment-friendly biocide. This method is significant for the future in the sense that it makes use of a natural product and causes less pollution, and its effectiveness does not decrease due to the gradually increased resistance of bacteria against many biocides. Killing microorganisms by photosensitizers and phototherapy is emphasized especially in the field of medicine. This method is expected to gain further significance for the leather industry in the course of time.

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