Disinfection of *E. coli* by UV-A radiation and the aid of hydrogen peroxide

Bachelor thesis

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Ο/η κάτωθι υπογεγραμμένος/η Άννα Χασιωτάκη του Χριστοδούλου, με αριθμό μητρώου 18679013 φοιτητής/τρια του Πανεπιστημίου Δυτικής Αττικής της Σχολής ΣΕΥΠ του Τμήματος Δημόσιας και Κοινοτικής Υγείας, δηλώνω υπεύθυνα ότι:

«Είμαι συγγραφέας αυτής της πτυχιακής/διπλωματικής εργασίας και ότι κάθε βοήθεια την οποία είχα για την προετοιμασία της είναι πλήρως αναγνωρισμένη και αναφέρεται στην εργασία. Επίσης, οι όποιες πηγές από τις οποίες έκανα χρήση δεδομένων, ιδεών ή λέξεων, είτε ακριβώς είτε παραφρασμένες, αναφέρονται στο σύνολό τους, με πλήρη αναφορά στους συγγραφείς, τον εκδοτικό οίκο ή το περιοδικό, συμπεριλαμβανομένων και των πηγών που ενδεχομένως χρησιμοποιήθηκαν από το διαδίκτυο. Επίσης, βεβαιώνω ότι αυτή η εργασία έχει συγγραφεί από μένα αποκλειστικά και αποτελεί προϊόν πνευματικής ιδιοκτησίας τόσο της δικής μου, όσο και του Ιδρύματος.

Παράβαση της ανωτέρω ακαδημαϊκής μου ευθύνης αποτελεί ουσιώδη λόγο για την ανάκληση του πτυχίου μου».

Ο/Η Δηλών/ούσα

ΧΑΣΙΩΤΑΚΗ ΑΝΝΑ

(Υπογραφή)

Summary

The growth of the population brings with it a series of factors that increase at the same time, one of the main ones being the consumption and use of water; developing countries are the most affected in terms of access to drinking water and safe sanitation, and it is reflected in the report generated in June 2019 by the World Health Organization (WHO) in conjunction with the United Nations Fund United Nations for Children (UNICEF) where it indicates that 2,200 million people in the world do not have drinking water services and 4,200 million people do not have safe sanitation.

As a result of this problem, organizations such as The Water Project, Charity: water, Water.org, International Water Association, among others, have emerged, which seek to create awareness about this resource, help access and seek solutions; and it is from the consumption and use of water that another problem is generated, such as the generation of wastewater.

The treatment of drinking water in developing countries, especially people with limited resources and rural areas, is carried out in the simplest way possible, cooking the water, but sometimes that money is preferred to invest in food as a priority, and it is from there they must find another way to obtain safe water.

Those interested in the subject began to look for a safe, easy to carry out and economical way, and realized that the sun could help, and from then on solar disinfection began to gain importance in research and yield favorable results in terms of pathogen inactivation.

Today the technique is called solar water disinfection – SODIS, which has already been used in more than 30 countries since 2001.

The main advantage of solar disinfection is the almost zero cost, but at the cost of long exposure times of water to solar radiation and in limited volumetric quantities (2 L plastic or glass bottles).

The main objective of this Final Degree Project is to seek improvements in solar water disinfection through the use of catalysts and oxidants, and the effects that these can have on the main pathogenic microorganisms, such as coliforms.

That is why, in order to carry out the study, bibliography was used on the aforementioned topics, in addition to venturing into advanced oxidation processes, which in recent times are being studied more and more, not only in order to achieve inactivation of microorganisms, but also to seek the best possible combinations, find the appropriate concentration of reagents, guarantee the lowest operating cost, avoid the formation of by-products, among many others.

Therefore, for our study it was decided to use hydrogen peroxide, being the second most oxidizing species (2.8 V) after fluorine.

The initial idea of the work was to establish different points within the bacterial life for their subsequent inactivation and to make a comparison between these points according to the determined processes, expecting results based on predetermined and controlled conditions (exposure time, concentration of catalysts, solar radiation) in addition to the action of the antioxidant defenses that bacteria present in their life phase.

- Study of the bacterial life cycle of E. coli, as the main indicator of fecal contamination;

- Study of bacterial disinfection with SODIS,

- Study of the disinfection of E. coli in its different phases of the life cycle for later comparison between them.

- Study of bacterial disinfection with SODIS in H_2O_2

Keywords

Solar disinfection, advanced oxidation processes (AOPs), *E. coli*

UNESCO Codes

2414.04 Bacteriology

2508.11 Water quality

3308.10 Wastewater technology

3308.11 Water pollution control

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4. Abbreviations

WWTP: Waste Water Treatment Plan

SODIS: Solar Disinfection

AOP: Advanced Oxidation Process

LB: Luria Britani

CFU/mL: Colony Forming Units per Ml

PCA: Plate Count Agar

5. Introduction

During the 21st century, the supply of drinking water to meet human needs and the protection of life-support functions of freshwater systems, have been a critical challenge. The limits of the renewable water are being exceeded, since the freshwater is used for agriculture and industrial reasons (Covich 1993, Postel and Carpenter 1997). This over-pumping, endangers the life of the aquatic ecosystems by decreasing 10% of the fish population and the extinction of advantageous species.

With population growth expected by 2030 (United Nations 1998) and the overuse of aquatic ecosystem services, three major risks of water scarcity appear. These are the commission of healthy alimentation by also considering the water constraints of agriculture, the maintenance of aquatic environment in a healthy state and the aversion political instability in international river basins. Under these reasons, it becomes urgent to activate a WWTP.

The urban or integral water cycle constitutes a series of stages from precipitation, collection, purification treatments, distribution, use, sewage, wastewater treatment and return to water bodies.

As a result of population growth in the world, the exploitation of this water resource so vital for life is increased, and in turn brings with it the generation of wastewater.

The exploitation of water is carried out based on the availability of the resource, this availability varies between countries, between seasons (drought and flood), between the economic resources for its treatment and it can be a safe water for consumption.

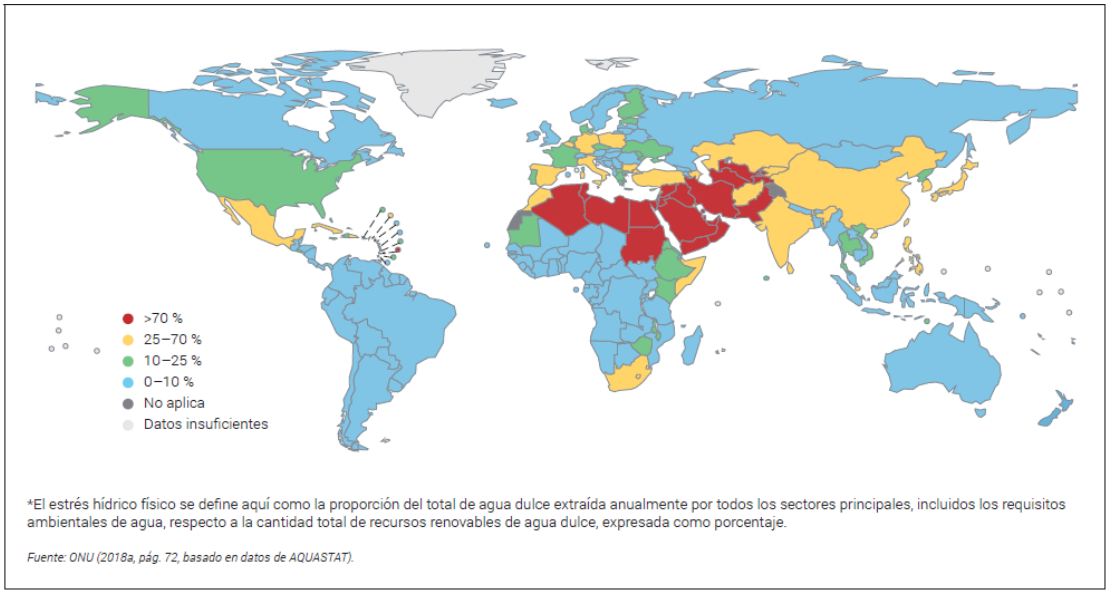

Image 1. Level of water stress in the world (United Nations World Report on the Development of Water Resources 2019).

The United Nations World Water Development Report 2019, with its central theme "Leaving no one behind", shows the global situation based on the level of water stress in Image 1.

The level of water stress represents the amount of water extracted in relation to the total amount of the resource; It is clearly observed in Figure 1 that the most affected countries belong to North Africa (Algeria, Libya, Egypt, among others) and South Asia (Saudi Arabia, Pakistan, Iran, among others), in addition, the 2019 Report mentions that this stress can be intensified by the effects of climate change.

Due to this, improvements should be sought over those traditional treatments such as solar disinfection or chlorination, even more so in those countries where the economy destined for water is not sufficient for it to be of this vital and important resource for access life to all people.

Solar disinfection is the easiest and simplest process, since it consists of placing the water in containers exposed to solar radiation and letting it act on it, taking into account environmental conditions such as temperature.

Some advantages of solar disinfection are practicality and almost zero cost, but with disadvantages such as the volumetric quantity to be treated (2 L plastic bottles), inappropriate natural factors and even possible bacterial regrowth.

If we take solar disinfection as a basis (operating conditions of temperature and solar radiation) but add catalysts and oxidants, we can convert it into an advanced oxidation process.

Photo-Fenton, is an advanced oxidation process that generates hydroxyl radicals (high oxidizing potential of 2.8 V) that occurs between iron with hydrogen peroxide with the addition of solar radiation.

Another improvement over solar disinfection, but which has not yet gained interest in its application, is the activation of persulfate, whose activation, whether due to factors such as heat, solar radiation or metals, generates sulfate radicals (oxidant potential 2.5 – 3, 1V).

6. Objectives

The main objective of this Final Degree Project is the study of water disinfection, focusing on bacterial inactivation through sunlight, and improving its effectiveness through oxidation processes.

To this end, the following specific objectives are presented:

- Learn and practice the methodology applied in microbiological analysis laboratories on water treatment issues.

- Determine the different phases of bacterial life through the design of the growth curve, with a more precise focus on the exponential phase and stationary phase of the bacteria.

- Study the characteristics and effectiveness of solar disinfection, seek its improvement with H_2O_2 processes

- Identify the responses of bacteria subjected to oxidative stress during advanced oxidation processes (AOP) according to the different phases of the life cycle.

- Compare the disinfection results

7. Materials

7.1 Bacteria

For all the tests carried out, wild strains of E. coli bacteria from wastewater were used. These strains were found in the Sanitary Engineering Laboratory of the Higher Technical School of Civil Engineers, Canals and Ports, they are considered as indicator species of faecal contamination due to the abundant and up-to-date information that is available on both and that allows extrapolation to other strains. Pathogenic bacteria.

The bacterial strains were stored at a temperature of -80°C, freezing reason to prevent their growth, this with the help of a protective agent, glycerol with a concentration of 20%.

Mother plates are prepared from one of the bacterial strains, which must be at room temperature and shaken for homogenization with glycerol. To do this, 20 µL of sample is spread in a 1/4 Petri dish with PCA (Plate Count Agar). For a correct dispersion and growth in the plate, perpendicular parallel lines must be drawn until covering the entire circumference of the plate. The mother board must be identified with basic information such as: board number, strain type and date. Subsequently, it is taken to the incubator for a period of 24 hours at a temperature of 37ºC. It is from there that the cultures of the strain can be made for the tests, taking into account that the mother plates can last up to 2 weeks for use.

To perform a culture (overnight, stationary phase), Luria Bertani (LB) must be prepared, a nutritionally rich broth suitable for bacterial growth, which must be heated and sterilized in the autoclave (duration 20 minutes at a temperature of 121ºC and 1.1 kg/m2 pressure). Subsequently, a colony from the mother plate is added in 5 mL of LB, and must be shaken at 200 r.p.m. with a temperature of 37 ° C in a time between 12 to 15 hours.

To study the different phases of bacterial growth, the same culture procedure mentioned above is carried out and the result is a function of the incubation time. To validate the life phase, the optical density of the culture is measured at 600 nm and the growth curve is plotted from the data.

Once the amount of bacteria in the culture has been reached and measured by the optical density of the sample, 1,000 µL of the sample is centrifuged at 7,000 r.p.m. for 2 min to separate the solid (bacterial) phase from the liquid (LB broth), cleaning with sterile saline (0.9% NaCl) is done in duplicate.

The solid phase (bacterial) is diluted in sterile saline solution, from which 100 µL are subsequently taken to mix with 900 µL of LB broth; From there, the optical density is measured again, data to start the test.

500 mL of distilled water (working pH between 6.3 to 6.8) is prepared in a sterilized and autoclaved bottle to be contaminated with the bacteria. Subsequently, it is separated into 50 mL samples for the dosage of the reagents to be applied in each advanced oxidation process; 2 series per process and test are performed.

7.1.1 Escherichia coli

Escherichia coli is a bacterium that belongs to the family Enterobacteriaceae considered facultative anaerobic, because it stains pink by Gram stain it is a Gram negative bacillus (Prescott et al., 2004).

It is bacillus-shaped and its average size ranges from 0.5 µm wide by 3 µm long. This bacterium is considered a normal flora microorganism since it is usually found in the human colon. Image 2 shows a microscopy image of this bacterium.

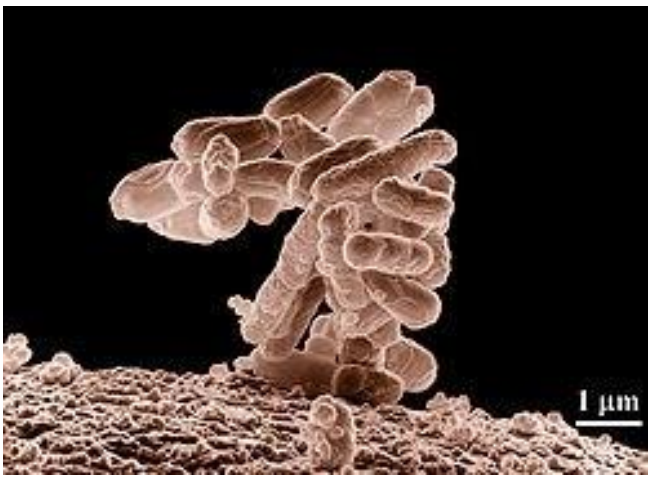

Image 2. Escherichia coli **(Wikipedia.org/wiki/Escherichia_coli)**

The E. coli bacterium is a prokaryotic microorganism (Prescott etc., 2004; Lizarbe, 2009) therefore, its structure and the functions of each one is as follows:

- Nucleoid: is a DNA molecule, which stores and transmits the genetic material.

- Ribosomes: they are spherical elements that provide a grainy appearance to the cytoplasm, in which proteins are synthesized.

- Cytoplasm: is the aqueous content (70-80%) that contains proteins, sugars, lipids, etc., in addition to the nucleoid and ribosomes.

- Plasma membrane: it is a thin permeable barrier (5 to 10 nm thick) that surrounds the cytoplasm, composed of lipids and proteins. It must fulfill several functions such as, being in contact with both the cellular environment and the outside, it allows the passage of molecules and ions; metabolic processes such as breathing, synthesis are developed; responds to chemicals through its special shielding molecules.

- Peri-plasmic space: it is made up of peptidoglycan which captures and transports nutrients, its estimated size is between 1 to 71 nm.

- Cell Wall: protects and gives rigidity, is located above the plasma membrane. The cell wall of a Gram negative bacterium (Image 3) is more complex than a Gram positive bacterium, because it is formed by a layer of peptidoglycan with a thickness between 2 and 7 nm, and in turn an outer membrane with a thickness between 7 and 7 nm. 8nm

- Flagella: they are long, thin and flexible filaments, which allow movement.

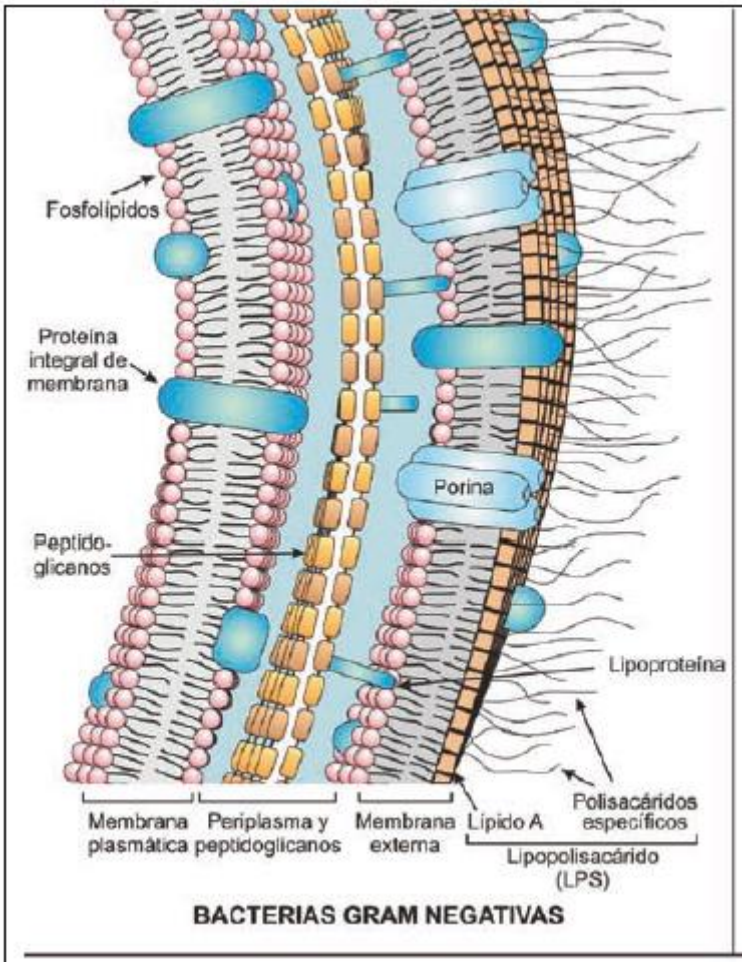

Image 3. Cell wall of a Gram negative bacterium (Lizarbe, 2009)

Its growth is based on the increase in cells, which increase logarithmically by binary fission, is measured in colony-forming units (CFU) and can be represented by a curve composed of four phases as shown in Image 4.

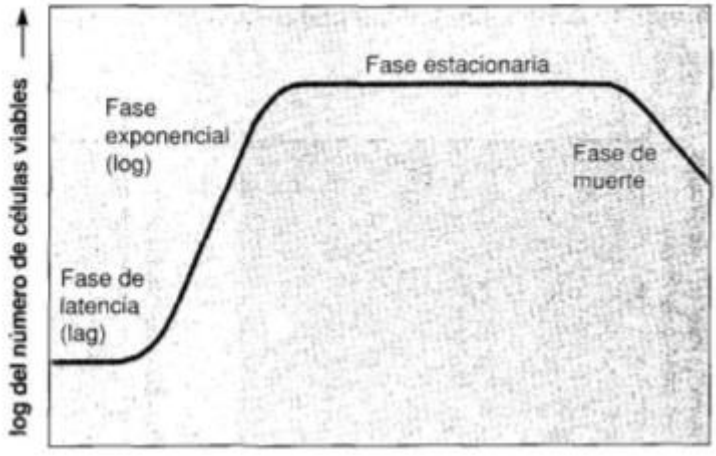

Image 4. Representation of the ideal E. coli growth curve (Prescott et al., 2004)

The first phase known as latency (lag) does not present an immediate increase in cells because it is a new culture medium and to which the initial bacteria must acclimatize. The duration of this phase is uncertain, since it can take from two hours to over five hours. The causes that affect the duration are the age of the bacteria (old, in other words), lack of nutrients in the culture liquid, or simply an alteration due to stress (Prescott, et al., 2004).

The exponential or logarithmic phase (log) is the one where the excessive growth begins depending on the culture medium, the environmental conditions and nutrients, and the genetics of the bacteria. This growth occurs at a constant speed, that is, the duplication is carried out at regular times until reaching its maximum level, which can be verified by the little variation between the amounts of bacteria from one time to another of each sample.

They are neutrophilic bacteria, their optimum growth pH is 7. If this value drops to values between 5.5 and 6, E. coli begins to synthesize proteins as a measure of response to an acid medium. Temperature is also a key factor for bacterial population growth (Prescott et al., 2004).

The stationary phase is considered when there is a balance between the multiplication and death of bacteria, reflected in the curve as a horizontal line. One of the causes for this phase to occur is the limitation of nutrients in the medium due to the excessive growth that comes from the exponential phase, in addition to the amount of residues that they themselves generate in the crop, which is considered toxic (Prescott et al. al., 2004).

Stationary phase bacteria present important physiological and morphological changes, due to the action of regulation genes in response to the stress to which they are subjected. Among the changes is the decrease in cell size and they pass to a more rounded shape. The thickness of the peptidoglycan of the cell wall, of the periplasmic space and with regard to the external membrane, also increases the amount of lipoprotein and lipopolysaccharides (Ramírez, 2005).

The bacterial growth curve ends with the endogenous or death phase, caused by the lack of nutrients in the culture and the excessive accumulation of its own residues. This phase is also considered logarithmic, that is, at constant intervals and quantities. To determine the death of a bacterium, it must be placed in a fresh medium, if it does not

grow or multiply, it is considered dead, since it does not have the ability to multiply (Prescott et al., 2004).

Bacteria must not only survive and reproduce as their main functions, but they must also know how to defend themselves against the conditions to which they are subjected, including repairing their damage, such as those caused by reactive oxygen species (ROS, from the English Reactive Oxygen Species) that they are formed from the metabolism of bacteria, which gives electrons to molecular oxygen (which in high concentrations can be toxic), as shown in Image 5 (Carvajal, 2018; Mishra and Imlay, 2012; Imlay 2008)

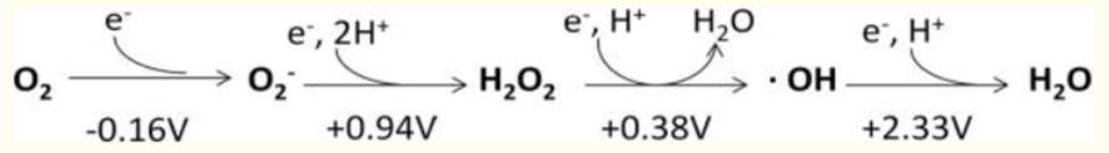

Image 5. Reactive oxygen species (Imlay, 2008).

The first reactive oxygen species obtained is superoxide, and from it the following, as presented below in equations (1 – 3):

$$2O_2^- + 2H^+ \xrightarrow{superóxido\ dismutasa} O_2 + H_2O_2 \quad (1)$$

$$2H_2O_2 \xrightarrow{catalasa} 2H_2O + O_2 \quad (2)$$

$$H_2O_2 + NADH + H^+ \xrightarrow{peroxidasa} 2H_2O + NAD^+ \quad (3)$$

Dismutase (equation 1) reduces intracellular hydrogen peroxide levels by catalyzing it into hydrogen peroxide and oxygen; catalase (equation 2-3) reduces hydrogen peroxide levels or catalyzes it in water by an intracellular reductant.

7.2 Reagent

Name	Chemical Form	Purity	Origin
Hydrogen Peroxide	H_2O_2	30% (p/p)	Sigma - Aldrich

7.3 Blends that were used

Saline Solution

In 1 L distilled water:

8 g Sodium Chloride – NaCl

0.8 g Potassium Chloride – KCl

pH regulation: among 7-7.5

Autoclave

Store in the fridge for max 3-4 weeks

Luria Britani (LB) Solution

In 1L distilled water:

10 g BactroTryptone

10 g Sodium Chloride – NaCl

5g Yeast Extract

Adjust to 7 – 7.5 (should be close)

Autoclave

Store in the fridge for max 3-4 weeks

Plate Count Agar (PCA)

In 1L distilled water:

20 g of PCA powder

Autoclave

Pour immediately to avoid solidification of the liquid

7.4 Auxiliary Equipment

Ultra Freezer

Equipment of the brand FRYKA was used to maintain the bacterial strains stored at a temperature of -80ºC for their conservation.

Autoclave

To keep the reactors, tools and samples sterile, a HERAEUS extractor hood was used.

Spectrophotometer

By a SELECTA brand spectrophotometer the absorbance of the samples was measured, verifying the bacterial concentration before performing a measurement to guarantee the initial load. Absorbance is the measurement of the initial concentration in a culture, through the absorption and scattering of light that bacteria carry out; therefore, the absorbed light is proportional to the bacterial concentration (Riverón-Rodríguez etc., 2011).

Suntest

To achieve the simulation in sunlight with constant variables, an ATLAS brand solar simulator was used with xenon lamps and ventilation, keeping the radiation intensity at 450 W/m2. The said solar simulator has a magnetic stirring plate, where the samples are placed, so as to have constant stirring inside the reactors.

Shaking Incubator

A shaking incubator of the brand Lab-Shaker was used for the growth of bacteria cultures

Drying machine

A HERAEUS brand equipment was used to dry the used accessories, once after the autoclave

Incubator stove

The contaminated plates were inserted into a SELECTA brand incubator oven, which distributes the same temperature in its interior.

Reactors

Needed to take into account the ability of maintaining the concentration of temperature inside the reactor, the material that were used during the solar disinfection experiments was UV-transparent pyrex glass. The volume of each reactor was 50 mL, with a diameter of 3.8 cm and was 7 cm high. The reactors were filled with Mili-Q water and bacteria were spiked inside to reach a concentration of 10^6 CFU/mL until being put in the UVA simulator. Once in the simulator, each reactor was being placed under magnetic agitation at 450 rpm along the experiment. Important note is that the pH of Mili-Q water is near neutral (pH=6.5). The reactors were sealed with aluminum foil to facilitate sample extraction. They must be previously sterilized in the autoclave to be used without risk of contamination of the samples. Additionally, those that had contact with chemical reagents must be washed with acid and rinsed with abundant distilled water, to achieve complete elimination of substances that can falsify the results.

Bacterial Strain

The bacterial strain used was E. coli K12 (MG1655), because, due to its non-pathogenic status, it makes it less risky to collect and cultivate in a laboratory. Furthermore, E. coli are a good indicator organisms of fecal contamination, are similar to the wild type and they often live longer than pathogens.

8. Methods

8.1 Solar Disinfection (SODIS)

Since ancient times, people have practiced solar disinfection of water without knowing that to date, it would save many people who do not have access to clean water for consumption; it can be estimated that more than 5 million people worldwide use solar disinfection - SODIS for water treatment (McGuigan et al., 2012).

By its name in English Solar Water Disinfection - SODIS, it is the current practice in developing countries of Africa, South America. This technique consists of placing water in plastic or glass containers, mainly in polyethylene terephthalate (PET) bottles. Subsequently, they are left to stand in the sun receiving UV rays for a minimum time of 6 hours, depending on the intensity of the light, as well as on whether the container is placed on a reflective surface or is covered in part of its area by reflective material. like aluminum. In this case, the exposure time would be less, since the bacteria are not resistant to the increase in temperature or to radiation for the exposure time that is needed. These circumstances make the cost negligible compared to other water disinfection processes such as chlorination, boiling, etc. Image 5 represents the steps to follow in the SODIS process (Lawrie et al., 2015).

Image 6. Steps to carry out solar disinfection – SODIS (Ecoinventos.com)

Solar disinfection inactivates microorganisms due to hydroxyl radicals (HO^*) formed intracellularly by exposure to solar radiation and Reactive Oxygen Species (ROS), which in turn eliminates the defense of antioxidant enzymes by exposure time (García-Fernández et al., 2019).

Solar radiation depends on the UV spectrum, being the most energetic UV-C (200 – 280 nm) which is retained by the ozone layer and the least energetic UV-A (320 – 400 nm) is the one that reaches the land surface; the inactivation of bacteria depends on the spectrum band as shown in Image 6. (Polo-López et al., 2013).

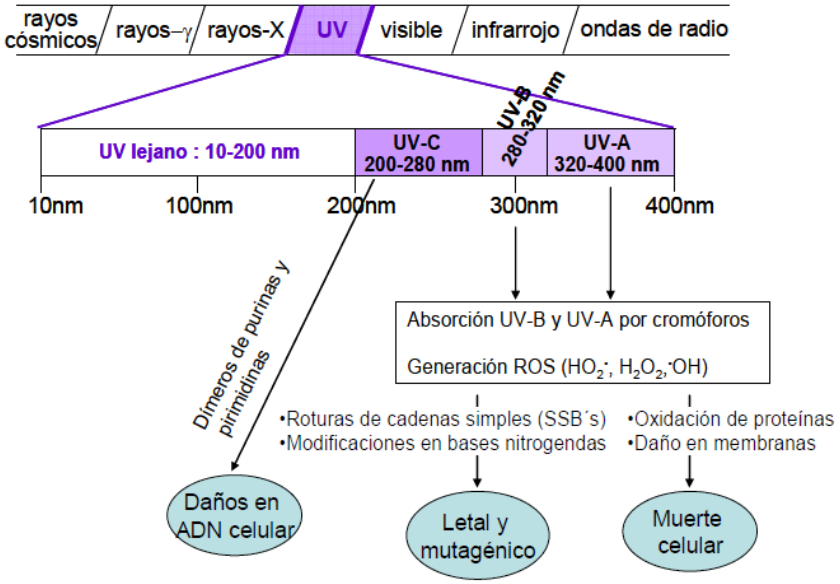

Figura 1.11. Principales daños biológicos producidos por las distintas franjas del espectro UV [Malato y cols., 2009].

Image 7. Damage to bacteria according to the UV spectrum band (Polo-López et al., 2013).

The thermal sensitivity of bacteria depends on the phase in which it is found within its life cycle, the information on results being variable between authors (Berney et al., 2006).

Solar disinfection - SODIS is a process which depends on the temperature of the water for the disinfection of bacteria, but total disinfection is more dependent on the solar radiation to which the containers are exposed (Berney et al., 2006).

This technique is defended and supported because it is sustainable, it can be done by anyone and the cost is almost zero; which makes it possible for the low-income population to access it.

In addition, this process can avoid dependence on solar radiation if it is supported with a UV dosimeter to complete the wave range and, in turn, will reduce the exposure time, but it is complicated since the investment in this equipment is considered expensive in the countries. on process of development.

Among the advantages of applying solar disinfection - SODIS in water is sustainability by using renewable energy such as sunlight, reusing plastic or glass containers to carry out the technique. It should be noted that the World Health Organization (WHO) approves and endorses its application. However, there are disadvantages such as a possible regrowth of microorganisms because the factors that influence during exposure do not damage the microorganisms enough and they can repair their DNA; the application of the technique depends on UV radiation and temperature which are natural factors; the waiting time is quite long in addition to being limited by the quantities to be treated.

It is from these and other associated disadvantages that the improvement of solar disinfection is sought through the use of catalysts and oxidants that help achieve a faster, more effective and efficient disinfection, without quantity limitations, among others, being known to currently as advanced oxidation processes.

8.2 Growth media and cultivation conditions

1 L of Luria – Bertani (LB) broth was prepared every week with Milli-Q water and sterilized in the autoclave. Pre-cultures of bacteria were prepared by taken 20 μL from the commercial bacteria and were spread onto the Plate Count Agar (PCA) plates. After 24 hours of incubation at 37^{o}C under O_2 environment in a Heraeus incubator, a colony was sampled and re-plated following the same method on a new plate and incubated again for 24 hours at 37^{o}C. Then the plate could be stored at 10oC for two weeks at maximum. For every experiment, one colony was picked and inoculated with 5mL of LB broth in sterilized PE Eppendorf flask of a 25 mL capacity. The incubation was taking place at 37oC and 180 rpm in an incubator shaker during 15-18 hours, until the cells reach the stationary phycological phase.

8.3 Sample preparation

2.000μL of the cells solution in the stationary phase were inoculated into two eppendorfs, with a capacity of 1.000μL each one, and then these two eppendorfs were centrifuged for 2 minutes at 180 rpm in a universal centrifuge. After centrifugation, the solid bacteria were found in the bottom of the eppendorfs, so the LB on the top was replaced with a new 1.000μL of saline solution in each, vortexed for 15 seconds and centrifuged again under the same conditions. The saline solution, this time, on the top of the eppendorfs was replaced with new 1.000μL of saline solution in each one, vortexed for 15 seconds. At the end of this process, having two eppendorfs well dispersed, one should be diluted in order to count the amount of bacteria in the suspension, by the use of a spectrophotometer. For the dilution, 100μL of the bacterial suspension with saline solution were inoculated into 900μL of clear saline solution in a new eppendorf. The new suspension was vortexed for 5 seconds and put in an 1 cm thick cuvette. Subsequently, the cuvette was put into the VR-2000 spectrophotometer which measures the concentration (optical density at 600nm) of bacteria that we have into this 10 times diluted suspension. This number should be multiplied by 10 in order to get the concentration C_1 of the original non-diluted. Having this concentration, the law of dilution $C_1V_1=C_2V_2$ is used, while V_1 is the volume needed to be injected into V_2 = 50mL in order to have a C_2 = 0,005 m/V concentration, which is translated to proximate 10^6 CFU/mL.

9. Results and Discussion

9.1 Bacterial life cycle – Growth phases

The determination of the growth curve of the bacteria *E. coli* was carried out starting from overnight cultures (growth in times between 12 and 15 hours) in a discontinuous culture of LB broth with constant process variables such as agitation (200 rpm), temperature (37ºC) and with a resulting optical density ranging between 4.0 and 5.0 for *E. coli*.

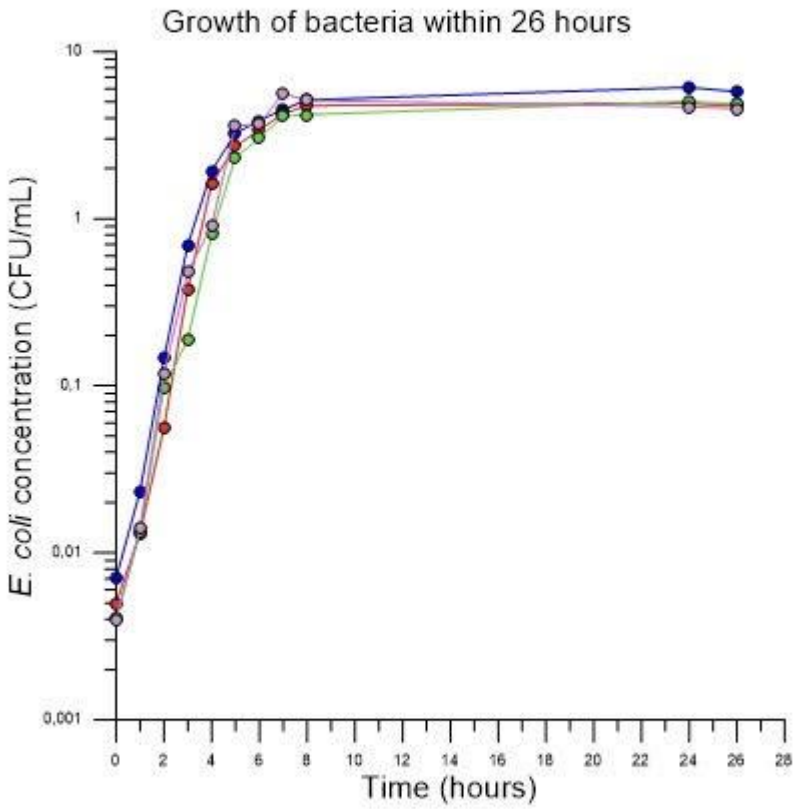

Image 8. Growing curve of E. coli within 26 hours

Image 8 shows the growth curve of the E. coli bacteria measured from the optical density.

The lag phase lasted less than one hour because of providing the best conditions for its growth: food availability, darkness and, ideal temperature for coliforms.

Two phases can be identified within the said curve:

i) the exponential phase was determined between 1 to 9 hours, taking off abruptly in the first hour since they had the necessary nutrients for their feeding and subsequent reproduction

ii) the stationary phase began after the 9th hour, where the concentration remained unchanged during the following measurements due to the

saturation of the culture medium, and equilibrium between growth and die-off of cells.

9.2 Inactivation of *E. coli* at stationary phase

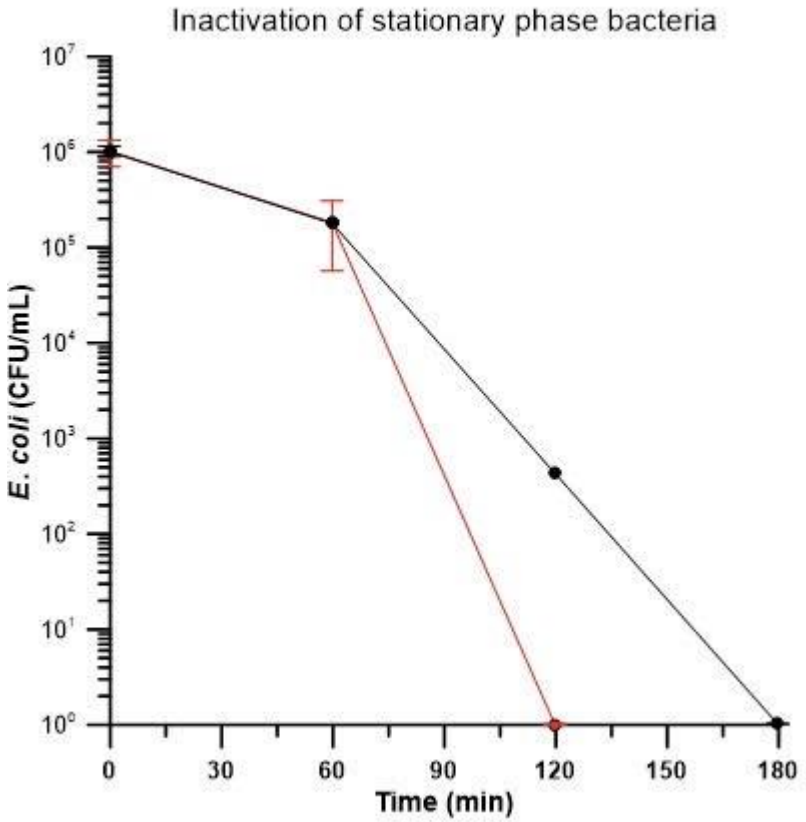

Image 9. Inactivation of E.coli with artificial UVA light

Image 9 shows the disinfection curve of *E.coli* bacteria in water starting from an overnight culture, through the use of UVA light determined in the present study, applying a radiation of 540 W/m2 and room temperature.

Here the bacteria originated from the overnight culture, i.e. they are stationary phase bacteria, which are found with optical densities (measures at 600nm) between 4.0 and 5.0, diluted to an initial OD_{600} of 0.005, that corresponds to 10^6 CFU/mL.

In our study that stress will be: ultraviolet radiation and hydrogen peroxide.

The effects of solar disinfection started being achieved by the first 60 minutes, but with a slow decline. After the first hour the bacteria started a fast inactivation with one sample being totally dead at the first 2 hours, while the other sample at the third hour. As a conclusion, the average time necessary to inactive 10^6 CFU/mL fluctuates around 150 minutes.

The disinfection curves show the decrease in the bacterial concentration with respect to time, in which 3 zones can be determined:

iv) zone 1 called shoulder, which shows us the resistance of the bacteria towards the stress to which they are subjected represented with a slow decline

v) zone 2 – exponential decay, which represents rapid death because the bacteria have reached their damage limit

vi) zone 3 – tail, which represents the culmination of cell death due to by-products originating from the reactions (Moreno et al., 2010; Marjanovic et al., 2018).

The duration of each zone is generally attributed to the variables involved in the process such as radiation, temperature, amount of catalyst and oxidants (for catalytic processes), agitation, reactors configuration, bacterial strain and the phase in which they are within their cycle of life, among others (Moreno et al., 2010).

Here the bacteria, which possess antioxidants defenses due to their enzymatic activity of catalase (mainly), they get inactivated by the stress to which they are subjected.

9.3 UVA inactivation of growing bacteria

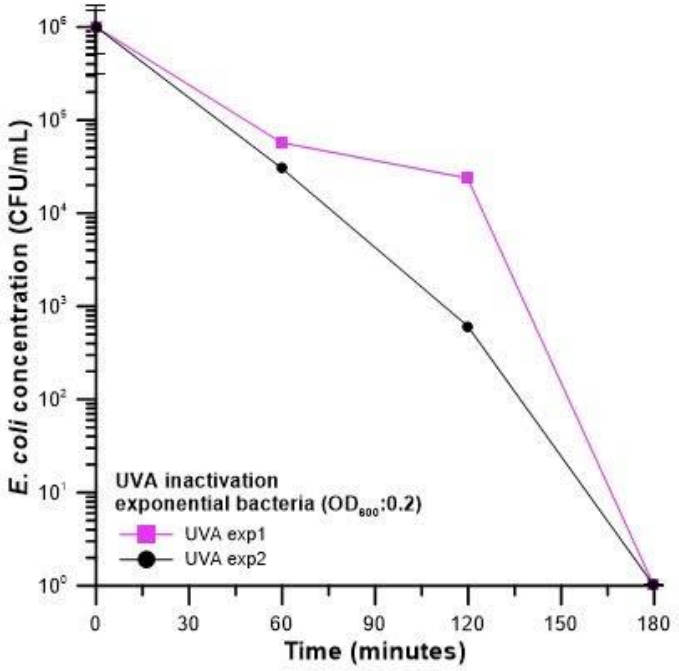

Image 10. Inactivation of exponentially growing bacteria (OD_{600}: 0.2) with artificial UVA light

The experiment of inactivating exponentially growing *E. coli* bacteria with optical density (600nm) 0.2, proves that the bacteria need more time in order to be killed than the overnight ones in the stationary phase. On the contrary, the time of fast decline starts at 120 minutes, while in the overnight bacteria at the very first hour.

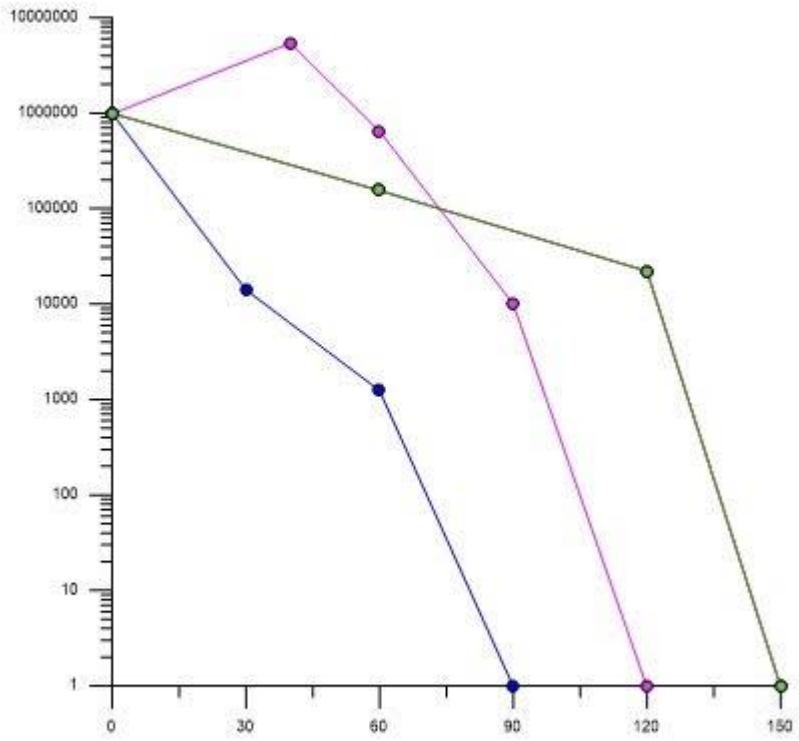

Image 11. Inactivation of exponential bacteria (OD_{600}: 0.4) with artificial UVA light

In this experiment of inactivating E. coli bacteria with optical density (600nm) 0,4 nm is observed that the bacteria reacted in three different ways to exposure in UVA light. On the one hand, the blue line started declining very fast. That means that the bacteria were easily affected by the artificial UVA light and started dying abruptly right after the first 60 minutes. On the other hand, the green line shows that bacteria needed more time to inactivate and started dying faster after the second hour of the experiment. Last but not least, in the pink line a periodic growth of bacteria is observed, while after the first hour, the bacteria start decreasing. Taking into account these results, it is proven that, despite the different reactions of each sample to the artificial UVA light within the first 60 minutes, all the bacteria are dead after the 150 minutes of their exposure.

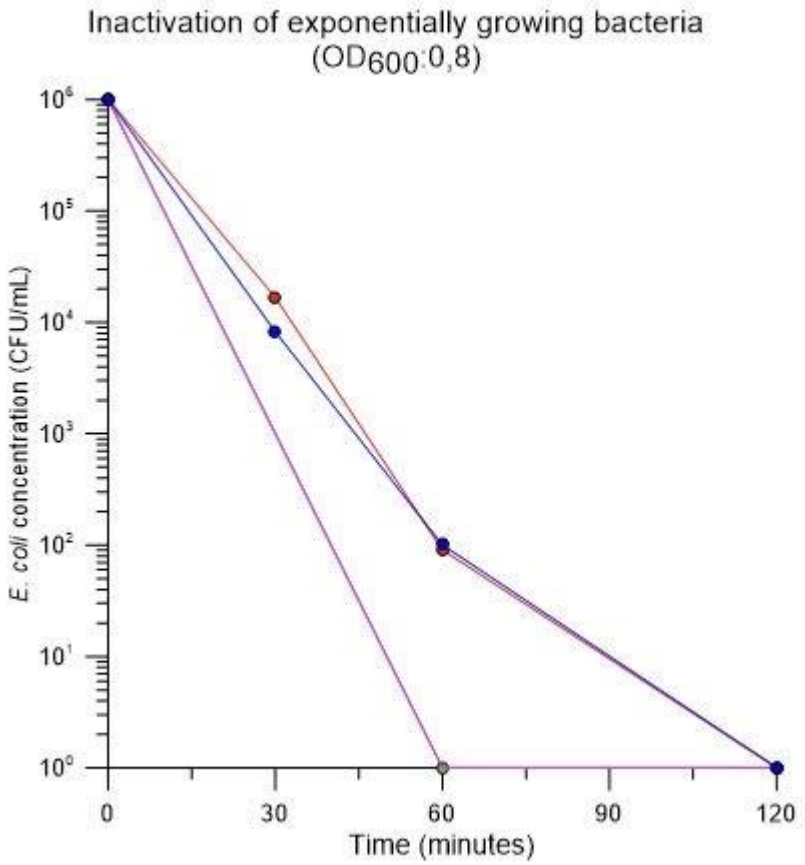

Image 12. Inactivation of exponential bacteria (OD_{600}: 0.8) with artificial UVA light

In this experiment of inactivating *E. coli* bacteria with optical density (600nm) 0,8 is observed that the bacteria got inactivated very fast by the artificial light. Within the first two hours the bacteria in all the samples were dead.

9.4 Results comparison

Taking into account the results of the previous mentioned experiments, it is proven that the fastest way to kill the bacteria *E.coli* under the effect of artificial UVA light, is during their exponential growth and more precisely, when they occur to have optical density (600nm) of 0.8.

9.5 Use of Hydrogen Peroxide for the disinfection of *E. coli*

Hydrogen Peroxide (H_2O2) could be used for disinfection with another disinfection alternative such as UV or ozone. So far, there are no full-scale systems built to utilize this disinfection method. On the experiments being conducted in this Thesis, the disinfection complement while using H_2O_2 is artificial UVA light (Black and Veatch Corporation, 2010).

In order to compare the mean lethal time of the bacteria with and without hydrogen peroxide, the experiments were repeated by adding 10 mg/L of H_2O_2.

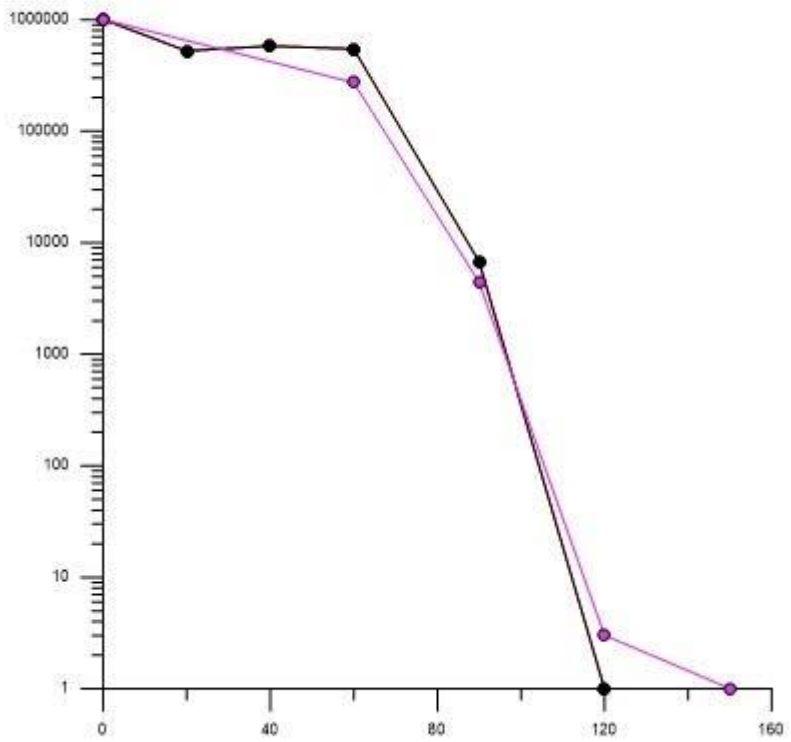

Image 13. Inactivation of E. coli in stationary phase under artificial UVA light with the assistance of 10 mg/L H_2O_2

In the experiment of inactivating *E. coli* under the artificial UVA light, the given results are that the first hour the number of bacteria was not affected. On the contrary, later, a rapid decrease in their population is observed.

For a deeper understating on the effect of the life cycle on bacterial inactivation by UVA in presence of H_2O_2, the experiments should be conducted with different phases of life of *E. coli*. For this, from the exponential *E. coli*, two different samples were taken. One with optical density (600nm) of the bacteria at 0.2 and one other at 0.8, as distinct phases of the growing cultures.

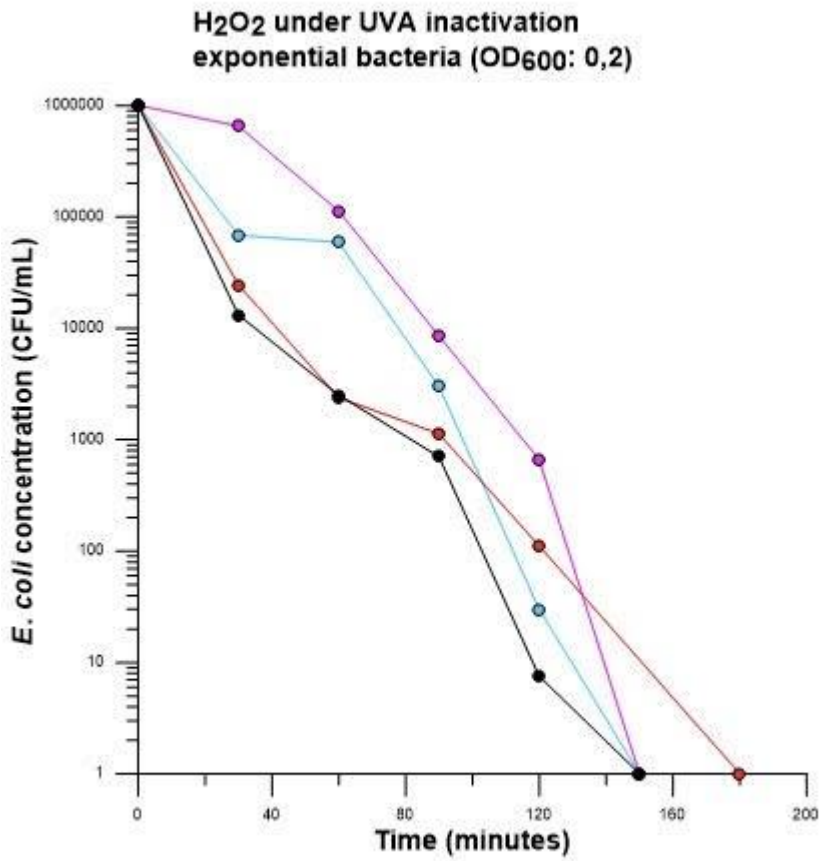

Image 14. Inactivation of E. coli with OD_{600}: 0,2 under artificial UVA light with the assistance of 10 mg/L H_2O_2

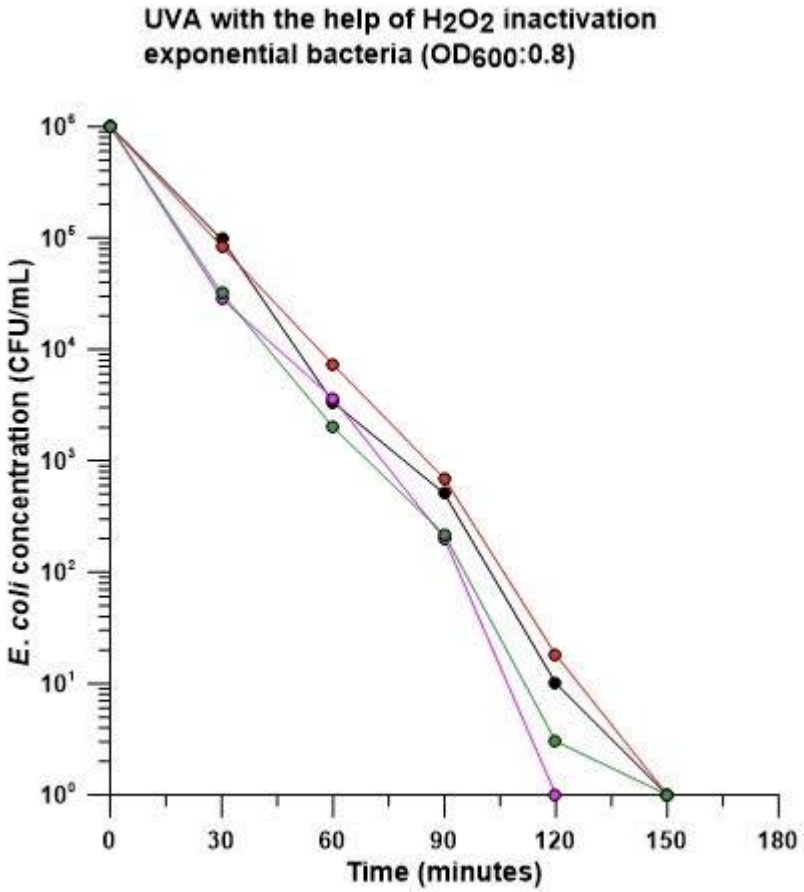

Image 15. Inactivation of E. coli with OD_{600}: 0,8 under artificial UVA light with the assistance of 30 mg L^{-1} H_2O_2

The results show that in three cases the bacteria are all inactivated after the first 150 minutes of the experiments. Only in the case of OD_{600}: 0.2 one sample takes longer to inactivate. These results prove the efficiency of hydrogen peroxide, as a disinfection factor, to accelerate the inactivation of *E. coli* under UVA light.

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