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Sterilization of Esherichia coli and the microorganisms of turmeric samples with corona discharge plasma

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Abstract

A low cost corona gun plasma system that works at atmospheric pressure and operates with high voltage power supply was tested for its biocidal effect on *Escherichia coli* bacteria as Gram-negative bacteria and different microorganisms of commercial turmeric (curcuma longa) as a combination of both Gram-negative and Gram-positive bacteria and results are compared. In these experiments, the *Escherichia coli* bacteria with a population of $4.4 \times 10^7\ cfu/ml$ were perfectly sterilized after 5 seconds irradiation of plasma generated by the corona gun, while it took about 80 s for sterilizing the microorganisms of turmeric samples. Results show that, with increasing the discharge energy and plasma exposure time, the rate of sterilization increases. Their survival curves are straight lines with two slopes for Gram-negative bacteria and three slopes for microorganisms in turmeric.

***PACs*:** 52.50.Dg; 52.75.Hn; 52.40.Hf; 52.77.Bn
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1. Introduction

Microbial sterilization is important in the biological and medical fields. Sterilization is based on either a physical or a chemical process that destroys or eliminates microorganisms, or both [1,2]. Traditional methods for sterilization include autoclaving, ovens, chemical such as ethylene oxide (EtO), and radiation (gamma rays), which are dependable and well understood. New techniques must deal with and overcome such problems as thermal sensitivity and destruction by heat, formation of toxic by-products, costs, and inefficiency in performance. However, traditional methods have their advantages as well as disadvantages. For these reasons, more rapid and less damaging method of sterilizing various materials is needed [3, 4]. A new sterilization method in the field of protection and conservation of materials from microorganisms is plasma treatment. In order to ensure easy use of such equipment and minimize the cost, researches in this field are concentrated on the atmospheric pressure plasmas using air as the working gas and necessary elements for killing bacteria will be formed in the plasma state [5-11]. Moreover, the advantages of corona plasma sterilization are the possibility of sterilization at a relatively low temperature, preserving the integrity of polymer-based materials, and it is safe as opposed to EtO. Moreover, it is not only capable of killing bacteria and viruses, but also capable of removing the dead bacteria and viruses (pyrogens) from the surface of the objects being sterilized.

In this method, two types of bacterial suspensions are exposed to the active species of a high voltage corona discharge generated at atmospheric pressure. Killing time of both samples has been investigated. The high voltage plasma operates at atmospheric pressure in air and produces antibacterial active species at room temperature.

Escherichia coli remain an important cause of diarrhoal disease worldwide [12]. *Escherichia coli* is a Gram-negative bacteria that, like many others, can cause an infection and is increasingly common in hospitals. Infection by *E.coli* bacteria results in significant public health problem worldwide. Spices, herbs, seeds and dehydrated vegetable substances bring a world of flavors, aromas and colors to food. Unfortunately, during their long journey to the table, spices and herbs become contaminated with bacteria and molds. Bacterial contamination may come from soil, insects, bird or rodent droppings or from the water used in processing. Fungal growth may occur before or during drying, or during storage or shipping. In any event, the consequences can be significant. Bacteria such as *Bacillus cereus* and *Clostridium perfringens* can cause a variety of foodborne illnesses.

Fortunately, most foodborne pathogens cannot survive modern sanitation measures. Even in case of extreme contamination, bacteria that remain after processing are unlikely to tolerate further cooking or heating. Left untreated, mold can produce aflatoxins, which destroy the flavor and color of the ingredients and make them unsuitable for use. No matter which sanitation tech-

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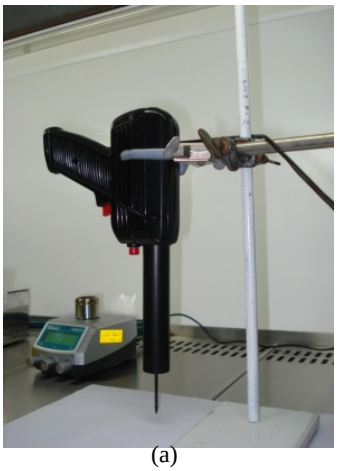
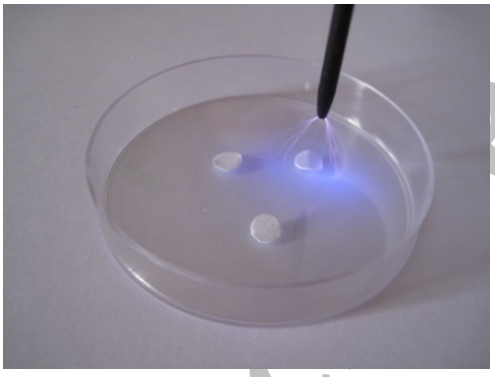
nology is selected, in order to prevent the contamination of finished food products it is important for dry ingredients to enter the processing area with very low bacterial and fungal counts. In most cases, only sterilized spices can meet the low plate counts specified by food processors manufacturing under HACCP or ISO standards.

This paper describes the sterilization effects on two types of bacterial suspensions, *escherichia coli* and turmeric samples using corona plasma at atmospheric pressure.



(a)



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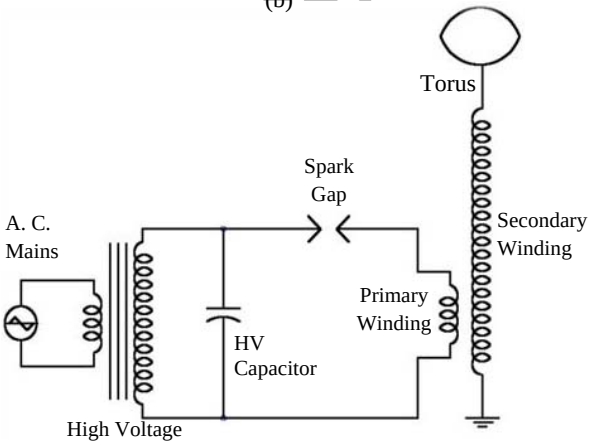

(c)

Fig. 1. Experimental setup a) atmospheric plasma corona discharge gun, b) an image of exposing the disks containing bacterial living cells to corona plasma, c) the inner structure of a simple Tesla coil.

2. Materials and methods

2.1. Experimental setup

The corona gun which is used in the experiments generates corona discharge. The corona gun is a simple Tesla coil that generates high voltage plasma. The output voltage of Tesla coil in corona gun was measured to be 1.5-1.7 kV. The plasma current in corona discharge was not measured but it can be assumed to be about 10^{-6} A [13]. The picture of the corona gun, an image of exposing the disks containing bacterial living cells to corona plasma and the electrical structure of the gun are shown in Fig. 1. The output of the gun is a multi streamer of plasma of about 2-5 cm length which is generated in air continuously.

The above described apparatus was placed in a Laminar Flow cabinet class II to prevent contamination. The above discharge system had an electrode which was kept nearly 1-1.2 cm above the impregnated disk surface for different samples.

In the case of *Escherichia coli* 5 μl of the cell suspension was transferred to each sterile paper disk. One of the disks inoculated with bacteria was kept as control (reference) which was not exposed to plasma. All of the disks except control ones were exposed form 0 up to 50 seconds to plasma. After plasma treatment, the disks that were exposed to plasma and control ones were put in 0.5 ml of 9% saline, and stirred for 60 s. After appropriately diluted, 100 μl of it was cultured onto nutrient agar plates. The number of colonies was counted after 48 hours of incubation at 30˚C. It should be noted that the distance between the plasma generating source and the disks inoculated with *Escherichia coli* was 1.2 cm.

For turmeric samples 5 μl of the cell suspension was transferred to each sterile paper disk. One of the disks inoculated with bacteria was kept as control (reference) which was not exposed to plasma. All of the disks except control ones were exposed for 0-10 and 0-100 seconds to plasma. After plasma treatment, the disks that were exposed to plasma and control ones were put in 245 μl of 9% saline, and vortexed for 60 s for removal of bacterial cells. After appropriately diluted, 200 μl of it was cultured onto nutrient agar plates. The number of colonies was counted after 24 hours of incubation at 30 ˚C. It should be noted that the distance between the plasma generating source and the disks inoculated with turmeric bacterial suspension was 1 cm.

2.2. Sample preparation

2.2.1. *Escherichia coli* sample preparation

First, from 24 h *Ecsherichia coli* Hb 101 bacteria, a preculture in Luria-Bertani broth media was prepared and 1% of it was inoculated into the 100 ml culture. Until the mid-logarithmic growth phase the bacterial culture should be incubated. In logarithmic growth phase cells reproduce rapidly and the time of cell divi-

sion is reduced to the shortest possible time. After incubating in incubator-shaker (with 150 rpm at the temperature of 37 ˚C), when bacterial growth reached to mid-logarithmic phase (absorbance rate of light in 600 nm is about 0.6), the culture contained approximately 10^{6} -10^{7} (cells / ml). Then the cells were suspended in 67 mili molar phosphate buffered solution (pH 7.0). After that the bacterial suspension was centrifuged with 8000 g for 15 minutes at the temperature of 4 ˚C.

The cells were washed two-three times by centrifugation at 8000 g for 15 min in a 67 mili molar phosphate buffered solution (pH 7.0) and resuspended in 30-40 ml of the same solution to give homogeneous cell density.

2.2.2. Turmeric sample preparation

From the turmeric sample, a suspension with dilution of 10^{-1} in sterile saline solution 9% with 0.1% tween 80 was prepared.

In order to isolate the microorganisms from the turmeric sample, first 9 g NaCl and 0.1g tween 80 in 100 ml distilled water was solved. Then 9 ml from this solution was poured in sterile test tubes and 1 g of turmeric was added to it and was well stirred. Thereafter, we waited for a while until turmeric was settled. At this stage of the work we used tween because tween causes the microorganisms to be isolated from turmeric and also isolates microorganisms from each other.

2.3. Colony counting

2.3.1. Colony counting of *Escherichia coli*

In order to determine the density of bacteria cells before exposing the samples to plasma in different stages of bacterial growth when bacteria were in an incubator-shaker, 1 ml of the culture was transferred into the cuvette in the spectrophotometer at different times.

The spectrophotometer at the wavelength of 600 nm, showed absorbance of light related to the growth of bacteria that it had reached. At each stage the bacteria were cultured. The spread method culture was used for cultivation. Then counting the density and diversity of microorganisms was performed by poure plate method. Thus the diagram of the number of cells versus absorption of light at different growth stages was obtained. From these data, the number of bacteria in a specific absorbance of light was found (from this table at the wavelength of 600 nm, for absorption of light of about 0.6, the number of cell showed the value of $4.4 \times 10^{7}\ cfu/ml$).

In order to evaluate the survivals after exposing the samples to plasma routinely 5 μl of the cell suspension was transferred to each sterile paper disk. All of the disks except control ones were exposed for 0-10 seconds to plasma. After plasma treatment, the disks that were exposed to plasma and control ones were put in 0.5 ml of saline 9%, and vortexed for 60 s for removal of bacterial cells. After appropriately diluted, 100 μl of it was cultured onto nutrient agar plates. Note that the paper disks were sterilized by autoclaving. The survivors were counted as colony forming units per each plate after incubation for 48 h at temperature of 30 °C.

2.3.2. Colony counting of turmeric samples

In order to evaluate the cells before exposing the samples to plasma, the density and diversity of the turmeric sample which was isolated in aqueous phase was counted by poure plate method. The dilutions from 10^{-1} to 10^{-6} were prepared. Then 100 μl from each of the above dilutions were cultured using spread method culture on nutrient agar medium (for each sample this process was repeated twice). Finally, the cultures were incubated for 24 h at the temperature of 30 ˚C. Each colony which was grown on the surface of nutrient agar medium was related to at least one bacterium and this way the number of live bacteria in the environment was calculated.

To evaluate the survivals after exposing the samples to plasma routinely 5 μl of the cell suspension was transferred to each sterile paper disk. All of the disks except control ones were exposed for 0-100 seconds to plasma. After plasma treatment, the disks that were exposed to plasma and control ones were put in 245 μl of sterile saline solution and mixed vigorously, for 60 s for separation of bacterial cells. After appropriately diluted, 200 μl of it was inoculated onto nutrient agar plates and cultured. Note that the paper disks were sterilized by autoclaving. The survivors were counted as colony forming units per each plate after incubation for 48 h at temperature of 30 °C.

3. Experimental Results

3.1.1 Experimental Results of *Escherichia coli*

In this study, paper disks of diameter of 5mm were used as target surface for corona discharge. Certain density of bacterial suspension (5μl) was transferred with a sampler to the paper impregnated disks. Then the bacterial suspension at fixed distance and in different time intervals exposed to plasma.

The disks were divided into 8 groups. The data obtained from experiments on *E.coli*. is shown in Table 1. For each group three repetitions were used. All stages of experiments were done for negative control, except that it was cultured without any treatment and no bacteria suspension was inoculated on the disk. In Fig. 2 we can see the nutrient agar plates after 48 h incubation. The plate 2a was not exposed to plasma contained 440 colonies (4.4×10^{7} CFU/ml), while the plate 2b was exposed to plasma for 1 sec contained 300 colonies (3.0×10^{7} CFU/ml) and the plate 2c was exposed to plasma for 5 seconds contains less than one colony.

Table 1. Data obtained from experiments on *E. coli*.

Group No.	Exposure time (s)	Density of the surviving microorganisms (cfu/ml)			Average density of the surviving microorganisms (cfu/ml)
		Sample 1	Sample 2	Sample 3	
first	0	4.4×10^7			4.4×10^7
second	1	0.3×10^6	1.2×10^7	3.0×10^7	1.4×10^7
third	5	< 1	< 1	< 1	< 1
forth	10	< 1	< 1	< 1	< 1
fifth	20	< 1	< 1	< 1	< 1
sixth	30	< 1	< 1	< 1	< 1
seventh	40	< 1	< 1	< 1	< 1
eighth	50	< 1	< 1	< 1	< 1
Negative control	0	< 1			< 1

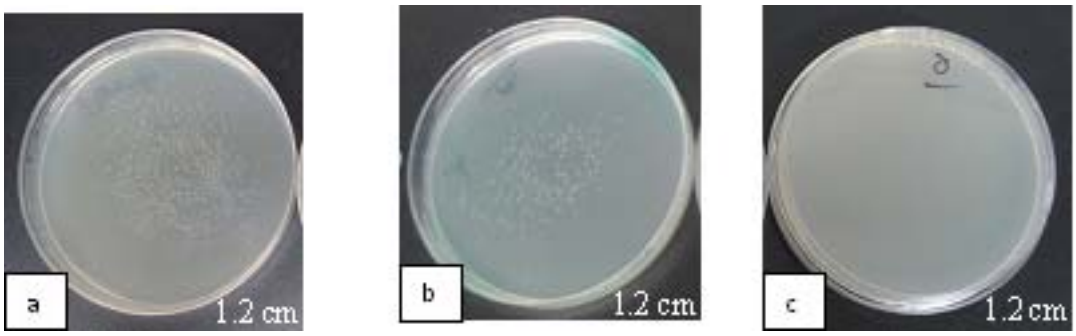

Fig. 2. Appearance of the nutrient agar plates after incubation; (a) reference plate contains 440 colonies (corresponding to 4.4×10^7 CFU/ml *Escherichia coli*), (b) after 1 s treatment contains 300 colonies; corresponding to 3.0×10^7 CFU/ml *Escherichia coli*, and (c) after 5 s treatment (contains less than 1 colony).

We found that the sterilization effect can be confirmed for all of three samples irradiated under the plasma discharges longer than 5. After incubation on nutrient agar plates during 48 hours, we judged whether the cells were perfectly sterilized or not. In Fig. 3, we show the experimental results of plasma sterilization of *Escherichia coli* using corona plasma. In Fig. 3, we plotted the successful sterilization data as 8.64-log reduction of microorganisms population of the samples which were exposed to plasma for less than 5 s. No bacteria cells were observed in the samples which were exposed to plasma for more than 5 s. So killing time of *Escherichia coli* species has been obtained within 5 seconds. It should be noted that all of the experiments were done at room temperature.

3.1.2. Experimental Results of turmeric samples

Experiments on bacterial suspensions of turmeric samples were done in two series. In the first series of experiments, paper disks of diameter of 5 mm were used as target surface for corona discharge. Certain density of bacterial suspension (5 μl) was transferred

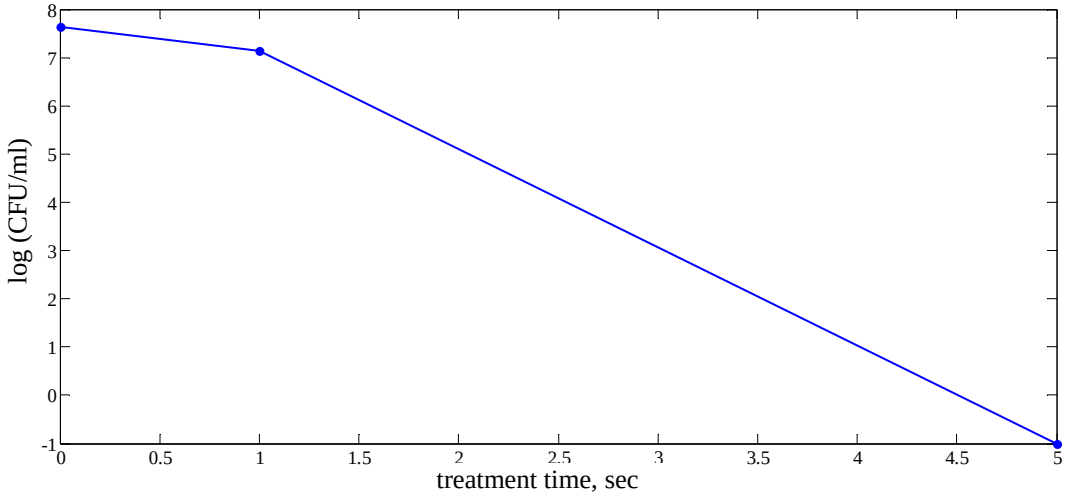

Fig. 3. Results of plasma sterilization of *Escherichia coli* with a population of $4.4 \times 10^7\ cfu/ml$.

with a sampler to the paper impregnated disks. Then the bacterial suspension at fixed distance and in different time intervals exposed to plasma.

The data obtained from the first series of experiments on turmeric samples is shown in Table 2. For each group three repetitions were used. As it can be seen from Table 2 the initial number of bacteria was about $1.6 \times 10^5\ cfu/ml$. All stages of experiments were done for negative control, except that it was cultured without any treatment and no bacteria suspension was inoculated on the disk. In Fig. 4 we can see the nutrient agar plates after 24 h incubation at 30 ˚C.

We found that the sterilization effect could not be seen for 10 seconds exposing the samples to plasma. The number of colonies that were grown on the surface of nutrient agar medium within 10 seconds of exposing to plasma was not reduced significantly. Thus in Fig. 4 only the plates belonging to 5 and 10 seconds of expo-

Table 2. Data obtained from the first series of experiments on turmeric.

Group No.	Exposure time (s)	Density of the surviving microorganisms (cfu/ml)			Average density of the surviving microorganisms (cfu/ml)
		Sample 1	Sample 2	Sample 3	
first	0	7.4×10^4	2.4×10^5	–	1.6×10^5
second	1	5.6×10^4	uncountable	6.4×10^4	6.0×10^4
third	2	7.5×10^4	3.6×10^4	7.2×10^4	6.1×10^4
forth	3	8.5×10^4	8.6×10^4	uncountable	8.5×10^4
fifth	4	2.9×10^4	2.1×10^4	5.4×10^4	3.5×10^4
sixth	5	5.5×10^4	3.1×10^4	4.2×10^4	4.3×10^4
seventh	6	2.3×10^4	4.0×10^4	5.4×10^4	3.9×10^4
eighth	7	2.4×10^4	2.8×10^4	3.4×10^4	2.9×10^4
ninth	8	1.4×10^4	3.6×10^4	3.8×10^4	2.9×10^4
tenth	9	2.0×10^4	3.5×10^4	7.8×10^4	4.4×10^4
eleventh	10	8.4×10^4	3.8×10^4	6.1×10^4	3.6×10^4
Negative control	0	< 1			< 1

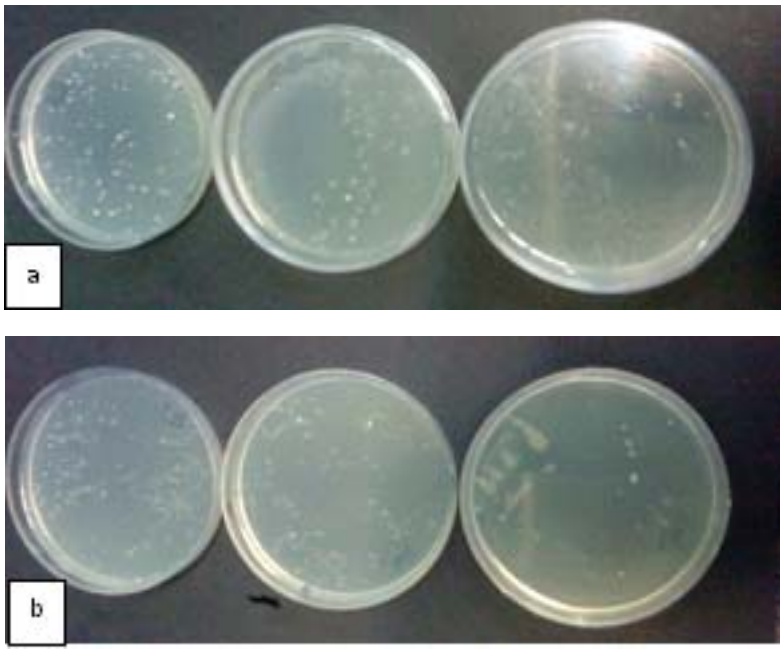

Fig. 4. Appearance of the nutrient agar plates after 24 h incubation at 30 ˚C. a) After 5 s treatment b) After 10 s treatment

sure to plasma are given. In Fig. 5, we show the experimental results of plasma sterilization of the turmeric samples using corona plasma. As it can be seen from the diagrams in Fig. 5(a) the density of microorganisms were not reduced significantly. The incubation results indicated positive after 24 hours culturing. Thus Killing of turmeric samples species has not been obtained within 10 seconds of plasma treatment.
The process of the second series of the experiments was the same as the first ones. Results of the second series of experiments of bacterial counts are shown in Table 3. For each group one repetition was used. As it can be seen from Table 3 the initial number of bacteria is about $2.4 \times 10^5\ cfu/ml$. In Fig. 6 we can see the nutrient agar plates after 24 h incubation at 30 ˚C.
we found that the sterilization effect could be observed for 80 seconds exposing the samples to plasma. In Fig. 5(b), as it can be seen from the diagrams the density of microorganisms were successfully reduced within 80 seconds. The incubation results for 80 s treatment with plasma indicated negative after 24 hours culturing. Thus killing time of turmeric samples species has been obtained within 80 seconds.
As experiments shows the diversity of microorganisms in turmeric target sample contained gram-negative bacteria and gram-positive bacteria. And also the molds *Aspergillus*, *Alternanarlia and Penicillium* have been shown in the target sample (see Fig. 7).

Table 3. Data obtained from the second series of experiments on turmeric.

Group No.	Exposure time (s)	Density of the surviving microorganisms (cfu/ml)
first	0	2.4×10^5
second	20	6.3×10^4
third	40	1.4×10^4
forth	50	8.4×10^3
fifth	70	2.0×10^2
sixth	80	< 1
seventh	90	< 1
eighth	100	< 1
Negative control	0	< 1

4. Discussion

In this work the atmospheric corona discharges generated by high voltage AC power supply is used to sterilize two types of bacterial suspensions, *Escherichia coli* and turmeric. The experiments aimed to find the optimal killing time of both microbial suspensions. Plasma produces specific active agents that have a biocidal effect of micro-organisms. During treatment the bacteria were exposed to the active atoms, molecules, charged particles and photons generated by the plasma. Although the biocidal mechanisms of plasma are still being investigated [14], it is believed that corona discharge plasma is produced by separating electrons from molecules (ionization) due to a high voltage between two electrodes or an electrode (anode) and water surface (cathode). Corona discharges appear

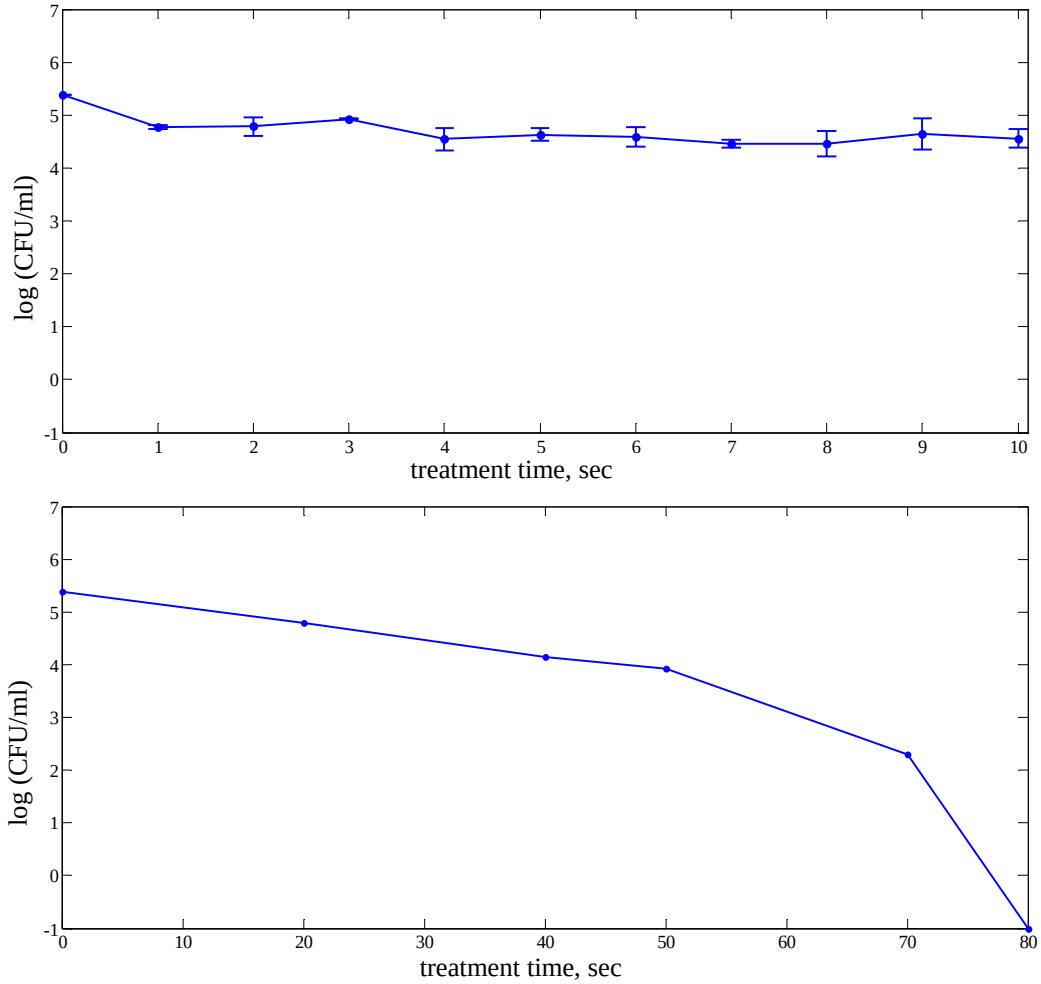

Fig. 5. Results of plasma sterilization for turmeric samples a) 0-10 s treatment. b) 0-100 s treatment.

as a luminous glow localized in the space around the electrode tip. When the voltage between the electrodes is several kilovolts, the electric field near the anode becomes very strong causing electron separation from the molecules. As the electrons move towards the anode, the positively charged ions (such as O^+) move towards the water surface and trigger chemical reactions to produce ozone, hydrogen peroxide and hydroxyls, which are known as active agents on microorganisms. In addition to such chemical formation near the water surface some UV light, although minimal, is also produced from corona discharge. Both the glow discharge plasma and produced UV lights are effective against a broad range of bacteria and other microorganisms [15].

The obtained results show the capacity even of a small plasma discharge to kill bacteria. The supposed elementary processes taking place in plasma produced by the high voltage discharge, is described by the following main reactions;

$$e + O_2 \rightarrow e + e + O^+ + O \quad (1)$$

$$O + O_2 + M \rightarrow O_3 + M \quad (2)$$

$$O^* \rightarrow O + hv \quad (3)$$

where the first reaction represents dissociation and ionization of molecular oxygen at electron–oxygen inelastic collisions while the second reaction represents the process of ozone generation. Both ozone (O_3) and active oxygen (O) are very reactive species. The last reaction represents the generation of UV light which also contribute to bacteria killing.

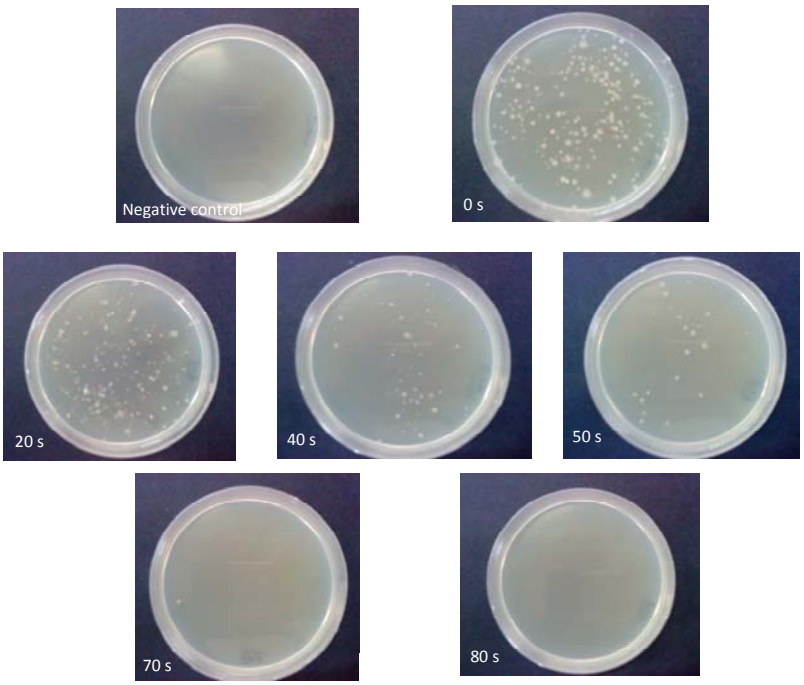

Fig. 6. Appearance of the nutrient agar plates after 24 h incubation at 30 ˚C. As the time of treatment increases, the number of colonies that are grown on the surface of nutrient agar medium decreases. As it can be seen, no colony was grown on the plate after 80 seconds treatment.

The minimum time to kill *Escherichia coli* cells was 5 seconds while the minimum time needed to kill of microbial cells in turmeric samples was about 80 seconds. Probably, the reason is that *Escherichia coli* is a gram-negative bacterium which is not much resistant to plasma treatment while the turmeric samples contain different microorganisms such as gram-negative and gram-positive bacteria and fungi with increased resistant to plasma treatment. The increased killing time of turmeric samples can be attributed to the existence of gram-positive bacteria which in general are more resistant bacteria. In fact, gram-positive bacteria in their sporulated form are more resistant than vegetative forms. The results of the experiments confirm the existence of more resistance bacteria in turmeric samples. When the turmeric sample suspensions exposed to plasma for exposure time less than 80 seconds, the plates still contained significant amounts of bacterial colonies as is shown in Fig. 4.

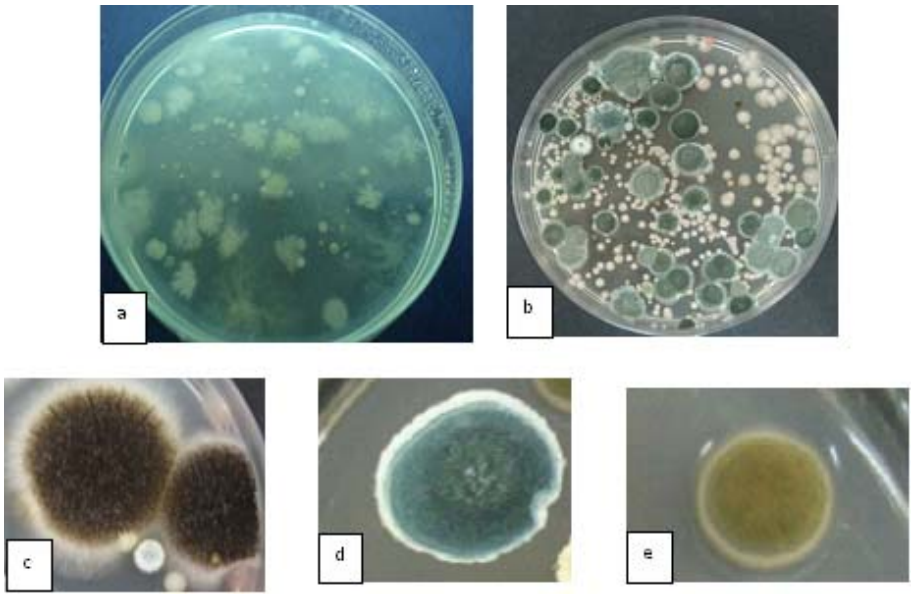

Fig. 7. a) Different separated bacterial colonies from the turmeric samples which were grown on nutrient agar plates b) Different separated mold colonies from the turmeric samples which were grown on sabouraud dextrose agar plates. c) Colonies of *Aspergillius*. d) Colonies of *Penicillium*. e) Colonies of *Alternanarlia*.

5. Conclusion

The effect of plasma sterilization potential of a non equilibrium high voltage plasma is studied. High voltage plasma is a non equilibrium plasma which has no time to achieve a local thermodynamic equilibrium. Fast electrons are generated in plasma, having enough energy to dissociate molecular oxygen or to produce ozone. The results obtained showed that plasma discharge system can kill both *Escherichia coli* and microorganisms of turmeric samples. Discharge energy and plasma exposure time are important parameters for inactivation of both samples in the plasma system. When the discharge energy and the plasma exposure time increased, bacteria killing time decreased or rate of cell killing increased.

The exposure of *Escherichia coli* which is a gram-negative bacterium to the corona plasma causes a total inactivation of over 8.64 logs in 5 seconds. Under the same condition, for turmeric samples the total inactivation time reduced 6.38 logs in about 80 seconds, because turmeric samples contain both Gram-negative and gram-positive bacteria as well as molds. Furthermore their survival curves are straight lines with two slopes for gram-negative bacteria and three slopes for microorganisms in turmeric.

The used discharge does not heat the surrounding objects or bacterial support and it is simple to efficiently kill the bacteria. Easy extension of this small size discharge can be realized by using a number of parallel discharge units.

The plasma system required much less exposure time than what has been reported in literature because of the more energetic particles in plasma and higher intensity UV radiation.

These results suggest that this sterilization method is easy to use, requires significantly less exposure time than other traditional methods. In addition, it is non-toxic. And the high voltage plasma at atmospheric pressure is a powerful sterilization tool for biological materials and condiments contaminated with microorganisms.

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