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Effect of ozonation on pathogenic bacteria

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ABSTRACT

In the present investigation of the human pathogenic bacteria such as *Escherichia coli,* and *Pseudomonas fluorescens, Salmonella typhi,* and *Klebsiella pneumoniae,* were analysed the ozone treatment by using ozonizer M221. To optical density was control and treated cultures were measured and to the different time intervals such as 5, 10 and 15 minutes of the optical density (by use of a digital spectrophotometer at 540nm) were gradually decreased. Among the treated bacterial species *E.coli* revealed high sensitivity to ozone treatment and compared to other bacterial strains. This was shown by the optical density value of *Escherichia coli,* and *Salmonella typhi; Klebsiella pneumoniae* and *Pseudomonas fluorescens* such as 0.81, 0.5.0.2 and 0.01; 0.290, 0.261, 0.245 and 0.221; 0.256, 0.254, 0.235 and 0.220; 0.219, 0.17, 0.08 and 0.045. The numbers of cells surviving after ozone treatments were less than for untreated cells.

Key words: Bacteria cultures, Ozonizer M221 and Centrifuge.

INTRODUCTION

Ozone is colourless gas that has an odor most often described as the smell of air after a spring electrical thunderstorm. Some people also refer to the odor as similar to the smell of water melons. Ozone (O_3) is an extremely constable gas. Consequently, it must be manufactured and used on site. It is the strongest oxidant of the common oxidizing agents. Ozone is manufactured by passing air (or) oxygen through two electrodes with high, alternating potential difference. Ozone is formed by a high energy input splitting the O_2 (oxygen) molecule. Single 'O' rapidly combines with available O_2 to form the very reactive O_3 (Baysan and Beighton 2007).

Ozone is formed by electric discharge through oxygen or air. It is also formed by ultraviolet (UV) radiation at 2,000 to 2,100A, especially in the higher atmosphere (40 km or 15 to 30 miles). The concentration at the surface of the earth is about 10^{-8} (parts by volume). Ozone absorbs UV at 2,537 and is decomposed (Benoit *et al* 1995). The deleterious effect of ozone on life is advantageous to man. Ozone kills bacteria and is used for this purpose in sewage disposal plants and in preservation of meat during the tenderizing process etc. In this latter instance, a part of the effect of UV lamps. In sterilizing is considered to be due to the ozone (0.1 ppm) produced by the wavelengths of 1,750 to 2,000 or 2,100A.

The concentration of ozone which kills bacteria has been variously reported to be 0.04 to 0.1 ppm (volume), whereas the toxicity for small animals is 3 to 12 ppm (Stockinger 1959). Humans experience headache, and most people can detect the odor at 0.02 to 0.04 ppm.

Ozone in the food industry has been investigated with regard to food preservation, shelf-life extension equipment sterilization and improvement of food plant effluent. In addition, ozonation has been used as a method for breathing

water in Europe. In France several municipal drinking water facilities have used ozone as the primary disinfectant since 1906. Effectiveness of clinical application of ozone gas remains controversial. Dahnhadt *et al* (2006) had shown an arrest of dentinal carries in children. Starting with a similar clinical situation we compared the immediate effects of gaseous ozone and of chlorohexidine gel on bacteria in activated curious lesions.

MATERIALS AND METHODS

Microorganisms or microbial culture such as *Escherichia coli, Pseudomonas fluorescens, Salmonella typhi* and *Klebsiella pneumoniae,* were obtained from Microbial Germ Plasm Culture Collection Unit (MGPCCU), Sri Gowri Biotech Research Academy, and Thanjavur and used for present investigation.

Preparation of Nutrient Broth

Nutrient broth (0.5g Peptone, 0.3g Beef extract, 0.5g Sodium chloride, and 100ml Distilled water) was prepared and then four bacterial cultures were inoculated into the test tubes containing and incubated at 37ºC for 24 hours. After inoculation, the cultures were characterized based on the colour, shape, size and margin of the colonies.

Gram Staining

A loopful of bacterial suspension was transferred on to a clean glass slide and smear was prepared. The smear was flooded with crystal violet for 1 minute. The stained smear was washed with tap water in order to remove excess stain. The smear was flooded with Gram's iodine for 1 minute and washed with tap water. Then the smear was decolorized with 95, ethyl alcohol. Add reagents, drop by drop until crystal violet fails to wash from smear washed with tap water. The decolorized smear was center stained with saffranin for 45 seconds and washed with tap water. The stained slide was dried and examined under the microscope.

Motility

A clean cover slip was taken and applied petroleum jelly on each of the four corners of the cover slip using a match stick. Then a loopful of culture was placed on center of the cover slip. The depression slide was placed on to the cover slip with the cavity failing down so that the depression covers the suspension. The preparations were examined under low power objective with reduced light.

Biochemical Analysis

Biochemical tests were performed according to standard methods.

Indole Test

Peptone broth was prepared and the bacterial culture was inoculated. After incubation, the indole, production was tested using Kovac's reagent. Red colour ring formation indicated positive reaction, whereas yellow colour ring indicated negative result.

Methyl Red Test

The bacterial culture was growing in MR-VP broth, and after the incubation it was added with methyl red indicator. Red colouration of the broth indicated, positive reaction, yellow colour development, indicated negative result.

Citrate Utilization Test

Sterile simmon's citrate agar slants were streaked with the bacterial culture and incubated at 37ºC for 24 hours. Change in colour from green to blue indicated positive reaction no colour change indicated negative results.

Urease Test

Sterile Christensen's urea slant were streaked with the bacterial cultures and incubated at 37ºC for 24 hours. Change in colour from yellow to pink indicated positive reaction.

Triple Sugar Iron Test

Triple sugar iron agar medium was prepared and made the slant with long butt. The culture was deep inoculated and streaking over the surface of agar in status. All tubes were incubated at 37ºC for 18 – 24 hrs.

Ozonizer Treatment
Nutrient medium was prepared and the four bacterial isolates were inoculated into the conical flask containing nutrient broth and incubated at 37ºC for 24 hours. Before ozone, the optical density of four bacterial broth cultures was determined by using spectrophotometer at 540nm. Ozone was generated from oxygen gas by a ozonizer M221 (Van Vijay plastics Pvt. Ltd., Pondicherry). The bacterial cultures were subjected to ozone treatment at various time intervals such as 5, 10 and 15 minutes using ozonizer. The OD values were taken at 540nm. Again the treated broth cultures were centrifuged at 10,000 rpm for 20 minutes and pellet were collected.

Growth Measurement
The pellets of four broth cultures (control and treated) were serially diluted with distilled water and the dilutions were made up to $10^{-7}$from 10^{-5} and 10 dilution 0.1ml of each suspension was spread on nutrient agar plates. The plates were incubated for 37ºC at 24 hrs. After incubation the bacterial colonies were counted and result were recorded.

$$\text{No. of cells} = \frac{\text{Organisms per millimeter}}{\text{Gram of the sample}} = \frac{\text{No. of colonies (Average of 3 replicates)}}{\text{Amount plated X dilution}}$$

RESULTS AND DISCUSSION

The results of morphological and biochemical analysis of bacterial cultures (*E.coli, Klebsiella pneumoniae, Salmonella typhi* and *Pseudomonas fluorescens*) were isolated (Table 1). The *E.coli* and *Salmonella typhi* culture was motile and *Pseudomonas fluorescens* and *Klebsiella pneumonia*e culture was non-motile. The following results were obtained from the present study of ozone treatment of pathogenic microorganism using ozonizer M221. To optical density of control and treated culture was measured and result was obtained as follows according to the time intervals 5, 10, and15 minutes. After treatment the OD was gradually decreased. Among the treated bacterial species *E.coli* revealed high sensitivity to ozone treatment and compared to other bacterial strains. This was shown by the optical density value of *Escherichia coli,* and *Salmonella typhi; Klebsiella pneumoniae* and *Pseudomonas fluorescens* such as 0.81, 0.5.0.2 and 0.01; 0.290, 0.261, 0.245 and 0.221; 0.256, 0.254, 0.235 and 0.220; 0.219, 0.17, 0.08 and 0.045 (Table 2).

Ozone is a potent oxidizing agent characterized by the ability to destroy a large variety of microorganisms, although its therapeutic application remains controversial (Benoit *et al.,* 1995). In the present study are in conformity with the above reports. It shows that ozone treatment effectively inhibit the growth of *E. coli* and *Pseudomonas fluorescens* respectively. Ozone gas to significantly reduce bacterial counts in infected dentine has recently been reported (Baysan and Beighton 2007).

The numbers of cells surviving after ozone treatments were less than untreated cells. The growth measurement also indicated the same reduction which was observed in the petriplates when the contact time of ozone in broth cultures increased gradually, the OD values and the number of cells decreased (Table 3). These results strongly suggested that ozone treatment for several minutes is able to disinfect.

Table 1. Biochemical Characterization

Biochemical Test	*E.coli*	*Pseudomonas fluorescens*	*Salmonella typhi*	*Klebsiella pneumoniae*
Motility	Motile	Non- Motile	Motile	Non- Motile
Grams staining	+	-	-	-
Indole	+	-	-	-
Methyl red	+	+	+	-
Voges proskauer	-	+	-	+
Citrate	-	-	+	+
Urease	+	-	-	+
Triple sugar iron	H_2S	Acid +Alkaline	Acid+Alkali	Acid +Alkali

Ozone gas is extremely effective for controlling bacteria and virus contamination Bacteria including *E. coli*, *Staphylococcus aureus, Salmonella typhimurium*, and sensitive to ozonated water under various conditions. The use of direct count method reveals that after disinfection, bacterial cells lose their ability to grow on culture media although they maintain a certain degree of activity (Desmont *et al* 1990).

Table 2. Ozone treatment on *E.coli, Pseudomonas fluorescens, Salmonella typhi* and *Klebsiella pneumonia*

Microorganism	Before Ozone Treatment	After Ozone Treatment		
	Control	5 min	10 min	15 min
E. coli	0.811	0.5	0.21	0.01
Pseudomonas fluorescens	0.219	0.178	0.086	0.045
Salmonella typhi	0.290	0.261	0.245	0.221
Klebsiella pneumoniae	0.256	0.254	0.235	0.220

Table 3. Number of colonies in nutrient agar plate

Microorganisms	Control	Ozone Treatment (min)		
		5	10	15
E. coli	120	100	40	14
Pseudomonas fluorescens	140	96	75	36
Salmonella typhi	150	120	80	32
Klebsiella pneumoniae	145	127	98	40

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