Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(3):7-12

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Optimization of ozonation through Weibull function for the complete inactivation of *Listeria monocytogenes*

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ABSTRACT

A study was performed to investigate the effect of ozone on the inactivation of Listeria monocytogenes when applied on solid media. Suspensions of L. monocytogenes ranging from 10^7 CFU/mL were inoculated onto selective medium, subsequently exposed to ozone in gaseous phase. The relationship between ozone concentration and treatment duration produced efficient changes in colony-forming units (CFU). Gaseous ozone effectively inactivates L. monocytogenes at concentrations of 0.55mg/Sec during short exposure times 30-300 seconds. Scanning electron micrographs were taken to examine the inactivation of bacterial cells. Weibull function was applied to predict the optimal ozonation duration required for the complete inactivation of L. monocytogenes. Accordingly, direct ozone diffusion treatment could be used as a prospective option to disinfect Listeria populations present in any solid medium.

Keywords: Inactivation, Ozone, *Listeria monocytogenes*, Weibull function, Gaseous phase

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INTRODUCTION

Listeria monocytogenes is a gram positive, foodborne pathogen that can grow in the range of 1° to 45°C and between zero and 10% water phase salt (NaCl). The organism is prevalent in the soil, water, sewage, and decaying vegetation. It can be readily isolated from humans, domestic animals (including pets), raw agricultural commodities, food processing environment, and the home. The organism is identified in many food varieties, especially meats, poultry, vegetables, dairy products, and fishery products [1-3]. Hence when consumed via contaminated food causes a serious disease called Listeriosis, where pregnant women, neonates, the elderly, and immunosuppressed individuals are more susceptible than healthy adults and children [4].

There is a need to develop innovative strategies for the control of Listeria in food and food processing environment and meet the consumer demand for minimally processed, ready-to-eat foods, without any loss in sensory and nutritional attributes [5].

Ozone is an allotropic modification of oxygen with a pungent characteristic odour. It is produced in the earth's atmosphere photochemically. Commercially, ozone is produced by activating oxygen in air with radiation of 185 nm emitted by high transmission UV lamps. The corona discharge method has been used most widely to produce higher concentrations of ozone[6-7]. Ozone is a powerful antimicrobial because of its progressive oxidation of vital cellular components.

As it is obligatory for researchers to perform valid studies to control the potential of food contamination, a study has been carried out to find the dose and duration of ozonation treatment required to inactivate *L. monocytogenes* suspensions dropped on a solid medium.

EXPERIMENTAL SECTION

Experimental Setup

The experimental setup consisted of an oxygen concentrator (Sim O_2 plus, Italy), ozone generator (Ozonetek Ltd., Chennai, India) with built-in oil-free compressor and reaction column. A controlled flow rate of 1 L/min of oxygen was used to produce 33mg/min of ozone. The ozonation chamber had a glass column of 300mm length and 120mm diameter. It was provided with a sample port at the center point. Teflon tube was used for connecting the ozone outlet port from the ozone generator to the ozone reaction chamber. The off gas was sent to the thermal destruction unit and converted to oxygen before venting out.

Inoculum Preparation

L. monocytogenes strains ATCC 19115, was obtained from Defence Food Research Laboratory, Mysore, India. *L. monocytogenes* was activated in tryptic soy broth (Hi Media, Mumbai, India) containing 0.6% yeast extract and grown in tryptic soy agar (Hi Media, Mumbai, India) at 30°C for 24h. After incubation, bacterial cells were harvested by centrifugation at 3,000 x g in a refrigerated (4°C) centrifuge and washed twice in 0.1 M phosphate buffer solution (pH 7) to a final concentration of 10^7 CFU/mL and were re-suspended in 1L of deionised water. Inoculum size was estimated by measuring absorbance at 600 nm (A600) using UV-Vis NIR Spectrophotometer (Shimadzu, Japan). This preparation made the day of ozonation and stored at 2-4°C.

Ozone treatment and Microbiological evaluation

10^7 CFU/mL of *L. monocytogenes* suspension was serially diluted to 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. PALCAM plates, a selective medium for *L. monocytogenes* were partitioned into four areas and 20 µL suspensions of each dilution were dropped onto each partition. Plates were exposed to ozone gas at a dose of 0.55 mg/Sec for duration of 30 seconds, 90 seconds, 120 seconds, 180 seconds, 240 seconds and 300 seconds without lids. After ozonation, plates were removed from the treatment chamber and then incubated for 48 h at 35°C. For each treatment combination, resultant bacterial growth (CFU/ mL) was determined along with a control plate without ozonation. All independent trials were replicated three times. Each microbial count was the mean of three determinations, expressed as CFU/mL.

Scanning Electron Micrograph

Inoculums before and after ozonation were fixed with 1% gluteraldehyde, followed by 1% paraformaldehyde and 4% osmium tetraoxide and were allowed to sit for 30 minutes at room temperature. After pipetting out the remaining fixatives, the bacterial cells were dehydrated in ascending concentrations (70%, 80%, 95%, and 100%) of ethanol for five minutes at each concentration. The materials were then placed under a hood and allowed to dry completely. The bacterial cells were mounted on aluminium stubs. The samples were examined under a scanning electron microscope (SEM, Make and Model: Icon Analytica; FEI Quanta 200). SEM was used to identify the morphology of the bacterial cells before and after ozonation (Figure 1).

Statistical analysis

Means of bacterial populations (CFU/mL) from each treatment were calculated from three replications for each experiment. Data were expressed as the means ± standard errors. Analysis of variance and Duncan's multiple range tests were performed to analyze the data using the SPSS statistical package (SPSS, version 11.0) and the significance of difference was defined at $P < 0.05$ and highly significant differences are defined as $P<0.0001$. The Experimental data was also fitted with the Weibullian model using Cox regression with survival model and hazard function. All models were fitted using IBM SPSS.

RESULTS AND DISCUSSION

An attempt of inactivating *L. monocytogenes* on solid media utilizing ozone in gaseous phase has been performed in this study, since ozonated water treatment at low concentrations could not produce effective inactivation due to the indiscriminate action of ozone to bacteria and other organic matter [8-9]. Exposure of media plates to gaseous ozone revealed to be successful in reducing bacterial levels. Eventually an increase in ozonation timing increased the inactivation of *L. monocytogenes.* It has proved that the inactivation efficiency increased by increasing ozone concentrations from 500 to 5000 ppm (1.07, 7.49 and 10.70 x 10^{-3} kg O_3/m^3) [10]. The survival rate of the colonies was linearly related to ozonation time. Complete inactivation in plates with partitions loaded with 20µL of 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} dilution occurred at 30 seconds, 180 seconds and 300 seconds of exposure to 0.55mg/sec ozone dose are given in Table 1 respectively.

Table 1. Enumeration of *Listeria monocytogenes* after ozonation

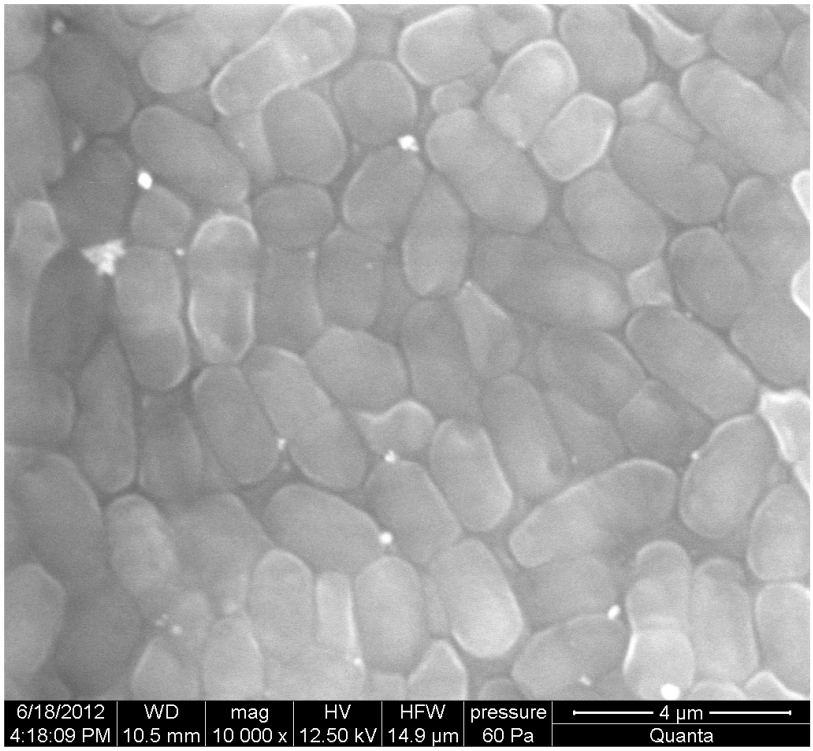
Ozonation Time (sec)	Survival colonies after Ozonation (CFU/mL)			
0	10^{-2}	10^{-3}	10^{-4}	10^{-5}
30	150	50	6	0
90	75	20	7	0
120	50	9	3	0
180	30	0	0	0
240	12	0	0	0
300	0	0	0	0

Table 2. ANOVA for the surviving colonies after ozonation

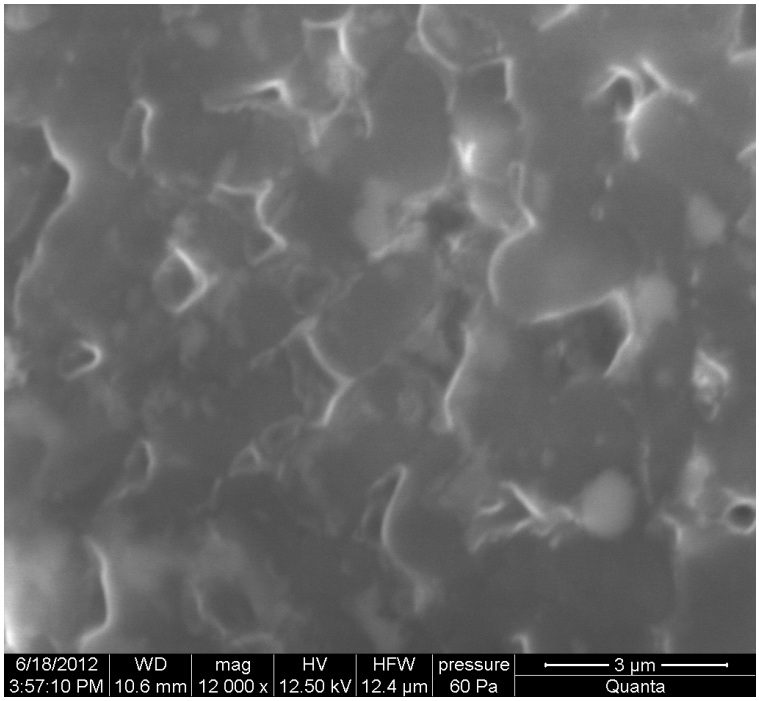
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	17930.167[a]	8	2241.271	3.451	.019
Intercept	7072.667	1	7072.667	10.891	.005
Ozonation Timings	7171.833	5	1434.367	2.209	.108
Dilutions	10758.333	3	3586.111	5.522	.009
Error	9741.167	15	649.411		
Total	34744.000	24			
Corrected Total	27671.333	23			

a. R Squared = .648 (Adjusted R Squared = .460)

When bacterial cells are subjected to ozone treatment, it disrupts the cell permeability by oxidizing the various components such as polyunsaturated fatty acids, membrane-bound enzymes, glycoproteins and glycolipids. Rapid death of the bacterial cells occurs when the double bonds of unsaturated lipids and the sulfhydryl groups of enzymes are oxidized by ozone [11-12] (Scott and Lesher 1963, Murray *et al.* 1965). Figure 1, apparently shows the scanning electron micrographs of damaged *L. monocytogenes* cells in the gaseous phase ozone treatment. A gaseous ozone dose of $0.1x10^{-3}$ gL^{-1} for 20 minutes was found to be the best time of exposure by Vaz-Velho 2001 to reduce the Listeria species to about 3.5 log $CFUmL^{-1}$[13]. While a gaseous ozone of 154 $x10^{-6}$ kg O_3 /m^3 270 $x10^{-6}$ kg O_3/ m^3 has been utilized for the complete inactivation of E. Coli and *Pseudomonas putida, Shewanella putrefaciens, Brochothrix thermosphacta, Enterobacter sp.* and *Lactobacillus plantarum* [14-15]. Even more than 10,000 ppm (2.14×10^{-2} kg O_3 /m^3) has also been used to inactivate salmonella in beef and poultry which is not approved to the colour and flavour of the targeted food[16]. For that reason this study utilized an appropriate dose of 0.55mg/sec for 300 seconds to completely inactivate a 20 µL suspension of *L. monocytogenes* at 10^{-2} to 10^{-5} dilutions dropped onto a solid media. As per the statistical analysis the number of colonies survived after ozonation had significant correlation with respect to dilution whereas not with respect to ozonation timings (Table 2).



a



b

Figure 1 Scanning electron micrographs of *Listeria monocytogenes* (a) before ozonation (b) after zonation seconds the surviving colony remains the same, hence the Weibull function predicts that there is no need to go for dilution till 10^{-5} power in experimental analysis. The model also predicts that entire colonies can be killed in 10^{-4} dilution itself (Figure 2 and 3)

As per Weibull analysis, the reduction in the number of colonies during exposure to 30 seconds, 90 seconds and 120 seconds of ozonation is found to be significant (Table 4). The Weibull model predicts that the 30 to 180 seconds is tending the infinity for iteration that is the serial dilution of (10^{-2} to 10^{-5}). Experimental results show that it needs 30 seconds of ozonation to inactivate 10^{-5} dilution whereas Weibull model fit the survival function and evaluated that it requires only 19 seconds to 20 seconds given in Table 3 and Figure 2 and 3. According to the Weibull function the colonies started decreasing once at 20 seconds and secondly after 43 seconds, between 20 and 43

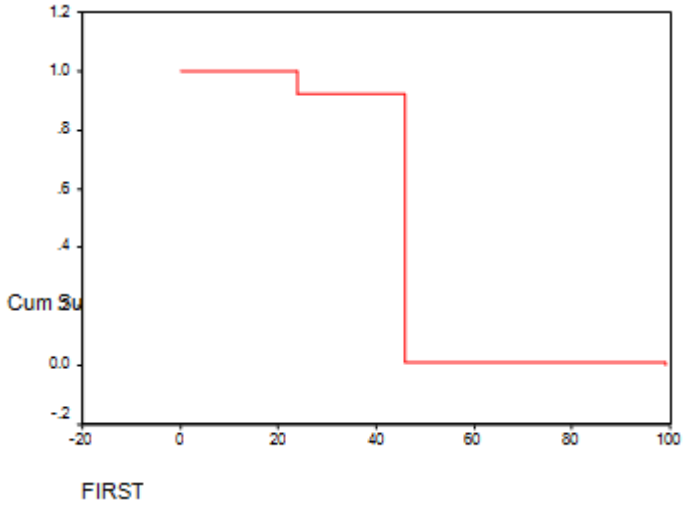


Figure 2 Survival Function of Colony Forming Units after Ozonation

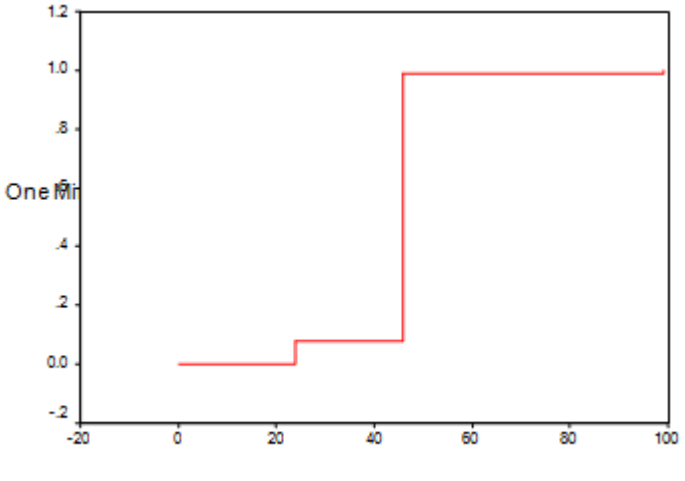


Figure 3 Hazard Function at Different Ozonation Timings

Though the experimental result predicts that 180 seconds is required to kill the entire *L. monocytogenes*, Weibull function has predicted that 120 seconds is sufficient to kill any dilution between (10^{-2} to 10^{-5}) Table 3. An Extreme level of serial dilution has been tried in the experiments so that the model can predict the required dilution. The power of function is normal till 23 seconds for 10^{-2} dilution and after 43 seconds only it starts killing the colonies for dilutions such as 10^{-3} and 10^{-4} respectively (Figure 2 and 3).

Table 3. Case Processing Summary

		N	Percent
Cases available in analysis	Event [a]	3	25.0%
	Censored	0	.0%
	Total	3	25.0%
Cases dropped	Cases with missing values	9	75.0%
	Cases with non-positive time	0	.0%
	Censored cases before the earliest event in a stratum	0	.0%
	Total	9	75.0%
Total		12	100.0%

a: Dependent Variable: FIRST

Table 4. Weibull Function (Ozonation timing and Colony Forming Units)

	B	SE	Wald	df	Sig.	Exp(B)
THER	-.700	1.360	.265	1	.607	.497
NINET	1.403	3.099	.205	1	.651	4.069

Ozone inactivates microorganisms in an environmentally friendly way through oxidization [17-18]. When applied at the appropriate dose to any food product it completely kills the microbes without affecting the nutritional quality of the targeted food [19]. Hence this research determines the optimal dose and duration of gaseous phase ozone exposure for the complete inactivation of *L. monocytogenes*.

CONCLUSION

The efficacy of ozone treatment was found to be a function of number of Colony Forming Units. Therefore, direct ozone diffusion treatment could be used as a potential alternative for control of *L. monocytogenes* populations in solid media or other solid foods. The dose and duration of ozone treatment can be optimized based on Weibull function The dose and duration of Ozone will vary based on the organic content of the target food and need not to be the same for all types of food items which can be optimized based on extensive laboratory studies and by the synergistic application of Weibull models.

Acknowledgements
This study is supported by DST-Govt of India in the form of DST-FAST TRACK young Scientist Scheme. The author is grateful to DRDO-BU CLS, Bharathiar University, Coimbatore, India for the facilities provided to execute the project.

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