CONTRIBUTION OF OZONE, pH AND TEMPERATURE IN THE INACTIVATION OF A FOODBORNE PATHOGEN *Listeria monocytogenes*– A STUDY USING RESPONSE SURFACE METHODOLOGY

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Abstract

Listeria monocytogenes is a Gram positive ubiquitous psychrotrophic bacterium responsible for foodborne infections worldwide. *L. monocytogenes* is capable of growing at refrigeration temperatures in high salt and acid foods. In this study a response surface methodology was used to determine the effects and interactions of pH (4 to 10), temperature (4 ºC to 40 ºC) and ozonation time (5 seconds to 60 seconds) on the inactivation of *L. monocytogenes* strains ATCC 19115. The models were validated on Phosphate buffered saline inoculated with *L. monocytogenes* (10^8CFU/mL) using a batch reactor. According to the study, the optimum pH and the temperature for the total inactivation of *L. monocytogenes* falls in the range of 4.5-7.5 and 4˚C-15˚C respectively. Analysis of Variance showed that the coefficient determination value (R^2) of *L. monocytogenes* inactivation was 0.9429. The study concluded that a 7 log reduction of *L. monocytogenes* can be successfully achieved by ozone exposure between 33 seconds and 49 seconds.

Keywords: *Listeria monocytogenes*; Inactivation; Temperature; Response surface methodology; Time; Ozone.

I. INTRODUCTION

Food-borne illnesses are rampant among the human population. Globally, the developed countries are searching for efficient and safe protocols for providing food safety. The best way to reduce incidence of food-borne diseases is to secure safe food supply. Although Hazard Analysis Critical Control Point (HACCP) system has been implemented in many food processing establishments, most outbreaks of food-borne illnesses still occur in the food service sectors including institutions, fast food restaurants, and food stores, where food products had undergone various treatments and considered as safe [1]. This situation indicates that hazards might still exist in the food supply systems. Human listeriosis is predominantly a food-borne disease caused by *L. monocytogenes*, and although rare, has a high mortality. The disease most often affects unborn or newly delivered infants, pregnant women, and the immunocompromised [2]. The pathogen has been consistently isolated from the production lines of fresh to cold-smoked fish [3]. A number of small outbreaks associated with smoked fish and shellfish have also been reported [4]. The survival of *L. monocytogenes* and other foodborne pathogens in apple, orange, pineapple, and white grape juice concentrates has also been studied [5].

Among the several disinfection processes available for the inactivation of food borne pathogens, the use of chlorine is banned because of the formation of organic halogens which ultimately cause environmental and ealth risks [6]. Moreover, there is a trend in eliminating chlorine from the disinfection process. Thus, there is a necessary for the usage of alternative sanitizer for conventional food

processors. A number of commercial fruit juice processors in the US and Europe started to employ ozone for pasteurization resulting in industry guidelines being issued by the FDA [7]. Ozone is an effective sanitizer with strong disinfecting properties. Ozone rapidly decomposes into oxygen, leaving no toxic residues making it environmental friendly [8]. Ozone decomposes producing numerous free radicals, predominantly hydroxyl free radicals which increase with increasing temperature and pH [9]. The mechanism of ozone self decomposition has been well documented [10-12]. Since ozone has a wide antimicrobial spectrum, which together with a high oxidation potential make it an attractive option for the food industry. Use of ozone has been reported for processing of various fruit juices including; apple cider [13] and orange juice [14]. Ozone surface treatment of fruit was reported to have minor effects on anthocyanin contents in strawberries [15] and blackberries [16] . There are numerous studies in the literature about the effect of ozone treatment on the safety and quality of iceberg lettuce, orange Juice, fresh cut green lettuce[17-18], Salmon – trout [19] and blackberry juice [20]. The use of ozone as a surface disinfectant of meats [21] and for the preservation of shrimps [22], broccoli sprouts [23] and Chicken [24] has been reported. Response Surface Methodology (RSM) is a statistical tool that includes the influence of individual factors as well as their interactive effects, hence it has been successfully used to model and optimize the ozonation process. It is employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations. A further benefit of using the RSM is the reduction of the number of experiments needed to compare a full experimental design at the same level [25]. In this research the variables such as pH, temperature and treatment time responsible for the inactivation of *L. monocytogenes* has been studied using ozonation process and the inactivation efficiencies were modelled using response surface methodology.

II. MATERIALS AND METHODS

A. Bacterial culture and preparation of inoculum

L. monocytogenes (ATCC 19115) were obtained from the Defence Food Research Laboratory in Mysore, India. *L. monocytogenes* were grown aerobically at 35 ºC for 24 h in Trypticase soy broth (TSB) (HiMedia, Mumbai, India) and stored at -70 ºC in TSB containing 10% glycerol (HiMedia, Mumbai, India). Prior to experiment, *L. monocytogenes* were propagated aerobically in TSB at 35 ºC for 18 h. *L. monocytogenes* cultures were streaked on Brain Heart Infusion agar (BHI, Hi Media, Mumbai, India) and incubated at 37ºC for 24 h. A single colony was chosen for further use, added into 50 mL tubes containing 10 mL of selective medium, and incubated overnight shaking at 37 ºC (150 rpm). 1mL of overnight culture was transferred to 100 mL of each fresh medium and was incubated at 37 ºC for 24 h. After incubation, culture was centrifuged at 2000x g for 15 min at 4 ºC. Cell pellets were washed twice in peptone water (0.1% sterile peptone, w/v). For each investigation, the cell concentration was further diluted in 1 L of sterile Phosphate buffered saline to yield a final concentration of 10^8 CFU/mL and then ozone treatment was applied.

Experimental Setup

The experimental setup consisted of an oxygen concentrator (Sim Oplus, Italy), ozone generator (Ozonetek Ltd., India) with built-in oil-free compressor and reaction column. A controlled flow rate of 2 L/min of oxygen was used to produce 2 g/h of ozone. The reactor had a glass column of 720 mm height, outer diameter of 45 mm, and an inner diameter of 35 mm and having a capacity to hold 1500 mL of a liquid sample. It was provided with a sample port at various points, an ozone gas inlet at the bottom with an air diffuser over the inlet port to diffuse the oxygen/ozone gas mixture through the column, and a closed top with a collection port to collect the unreacted ozone gas venting out. Teflon tube was used for connecting the ozone outlet port from the ozone generator to the ozone reaction chamber.

B. Experimental Design-Central Composite Design (CCD)

Response surface methodology (RSM) is a collection of statistical tools and techniques for exploring an approximate functional relationship between a response variable and a set of design variables [26]. (Myers and Montgomery, 1995**)**. A three-level factorial design was established with the help of the Design Expert software

(Central Composite Design Expert Version 8.0.3, Stat Ease, Minneapolis, USA). In the experimental design, model parameters were estimated by forming an optimal plan matrix using a second-degree quadratic polynomial equation:

$$Y = B_o + B_1X_1 + B_2X_2 + B_3X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 \quad ...(1)$$

Where Y predicts response (inactivation), Bo the constant coefficient , B_1, B_2, and B_3 the linear coefficient, B_{11}, B_{22}, B_{33} the quadratic coefficient, B_{12}, B_{13}, B_{23} the cross-products coefficient, and X_1, X_2, *and* X_3 were input variables. The variables and their levels are designated as -1.682, -1, 0, +1, and +1.682 (Table 1). According to the equation (1), it was found that a total of 20 runs are necessary to optimize the *L. monocytogenes* inactivation. Adequacy of the proposed model was then revealed using the diagnostic checking tests provided by analysis of variance (ANOVA). The quality of the fit polynomial model was expressed by the coefficient of determination, R2, adjusted R^2, and "adequate precision". The fitted polynomial equation was expressed as three-dimensional (3D) surface plots to visualize individual and interactive effect of variables on the response within the design range.

Table 1: Level of factors and their values used for the experiment

Variables coded values	Actual values of the coded values				
	1.682	−1.000	0	+1.000	+1.682
pH (X_1)	4	5.3	7	8.7	10
Temperature (X_2) ˚C	4	11.299	22	32.70	40
Ozonation time (X_3) Sec	5	16	32.5	49	60

C. Experimental Procedure

One liter of Phosphate buffered saline mixed with culture was transferred into the laboratory-scale batch reactor. The sample was then transferred to the ozonation chamber, and 66.6 mg O_3/min was passed into the chamber. The experiment was carried out as per the order given in Table 2. After treatment, the sample was transferred into conical flask sealed tightly with a rubber cork for further microbiological analysis

Table 2: The design matrix and observed values of the central composite design

Run Order	pH (X_1)	Temperature (X_2) (°C)	Ozonation Time (X_3) (Seconds)	Survival Organisms (after ozonation) (Y) CFU/mL
1	5.30	11.00	49.00	0
2	5.30	11.00	16.00	2
3	8.70	11.00	49.00	12
4	5.30	33.00	16.00	21
5	7.00	22.00	5.00	4
6	7.00	4.00	32.50	0
7	8.70	33.00	16.00	14
8	8.70	11.00	16.00	10
9	8.70	33.00	49.00	5
10	7.00	22.00	32.50	0
11	7.00	22.00	32.50	0
12	7.00	22.00	32.50	0
13	10.00	22.00	32.50	32
14	5.30	33.00	49.00	0
15	7.00	22.00	32.50	0
16	7.00	22.00	32.50	0
17	7.00	22.00	60.25	0
18	7.00	40.00	32.50	3
19	4.00	22.00	32.50	12
20	7.00	22.00	32.50	0

D. Microbiological analysis

After ozonation, 1mL of the sample in triplicate were evenly spread on the surface of the Listeria selective agar PALCAM (Hi Media, India) supplemented with modified Listeria selective supplement (FD061, Hi Media, India) plates aseptically. Each plate was incubated at 37°C for 48 h respectively. Each microbial count was the mean of three determinations, expressed as CFU/mL.

III. RESULTS AND DISCUSSION

A. Statistical Analysis and Fitting of Second-Order Polynomial Equation

Response surface methodology is an empirical modeling technique, where the relationship between a set of controllable experimental factors and observed results are evaluated [27]. Quite a number of factors persuade the inactivation of *L. monocytogenes*, of which pH, temperature and ozonation time plays a vital role. In order to study the combined effect of these variables, experiments were performed in different combinations using statistically designed experiments. The results of response for survival of the organism after inactivation (Y_1) are shown in Table 2. To decide the adequacy of various models to represent the inactivation efficiency, two different tests, namely the sequential model sum of squares and model summary statistics were carried out and the results are given in Table 3 and Table 4.

Table 3. Analysis of variance (ANOVA) for the fitted quadratic model

Source	Coefficient estimate	Sum of squares	DF	Mean square	*F* value	Prob >*F*
Model		1359.39	9	151.04	18.34	0.0001
Intercept	96.90					
X_1	-33.49	195.23	1	195.23	23.70	0.0007
X_2	+1.72	32.43	1	32.43	3.94	0.0753
X_3	-0.32	98.77	1	98.77	11.99	0.0061
$X_1 \times X_2$	-0.15	60.50	1	60.50	7.35	0.0219
$X_1 \times X_3$	+0.07	32.00	1	32.00	3.89	0.0770
$X_2 \times X_3$	-0.02	112.50	1	112.50	13.66	0.0041
X_1^2	+2.62	824.50	1	824.50	100.11	<0.0001
X_2^2	+2.61	1.44	1	1.44	0.17	0.6848
X_3^2	+1.81	3.50	1	3.50	0.42	0.5292
Residual		82.36	10	8.24		
Lack of Fit		82.36	5	16.47		
Pure Error		0.000	5	0.000		
Cor Total		1441.75	19			

Table 4. Model Summary Statistics

Source	S.D	R^2	Adjusted R^2	Predicted R^2	PRESS
Linear	8.35	0.2264	0.0814	-0.3172	1899.05
2FI	8.37	0.3686	0.0772	-0.3774	1985.87
Cubic	0.74	0.9977	0.9928	0.4963	726.16

In the model of inactivation of *L. monocytogenes* (*Y*) the probability value of <0.0001 implies that this model was significant. To test the estimated regression equation for the goodness of fit, Fisher's F-test was employed and the multiple correlation coefficient R^2 was calculated. The model F-value 18.34 implies that the model is significant for inactivation of *L. monocytogenes*. The ANOVA results for the response *Y* (survival of organisms), showed that the significant ($P < 0.01$) model with high R^2 value of 0.9429. Quadratic model was found to be maximum in adjusted R^2 (0.8915). Hence, quadratic model was chosen for further analysis. The lack of fit (LOF) F- value of this model implies the variation of data around the fitted model and shows significance. The experimental results were evaluated and the response, survival of organisms after treatment were obtained in the form of following regression equations (Eq.2),

Survival of *L. monocytogenes* (Y) = + 96.9017 - 33.4994 (pH) + 1.7261 (Temp) - 0.3252 (Time) - 0.1471 (pH*Temp) + 0.07130 (pH*Time) - 0.0206 (Temp*Time) + 2.6172 $(pH)^2$ + 0.0026 $(Temp)^2$ +0.0018 $(Time)^2$… (2)

Data were also analyzed to check the normality of the residuals. A normal probability plot of the residuals is shown in Fig 1. in which the actual values are the measured response data for a particular run and the predicted values are evaluated from the model and generated by the approximating the functions. A normal probability plot indicates whether the residuals follow a normal distribution, in which case the point will follow a straight line. The data points on the plot lie reasonably close to a straight line and it concludes that the data is fairly distributed.

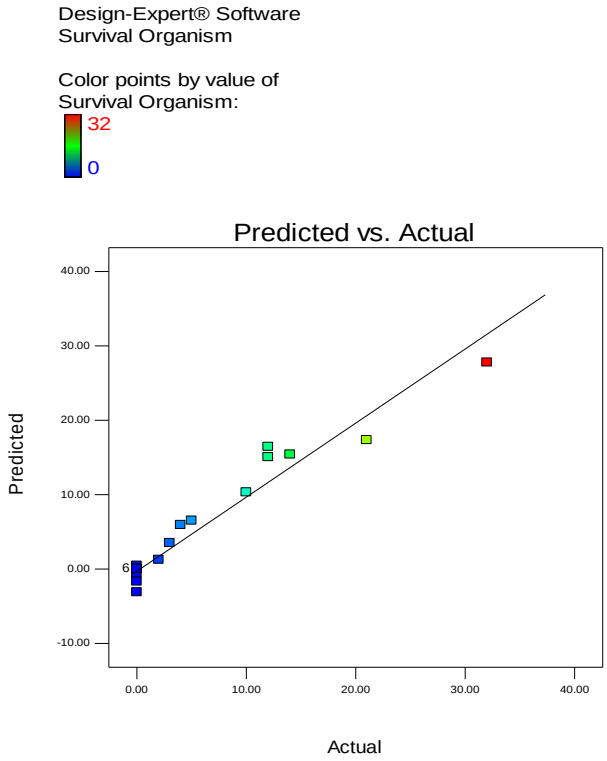


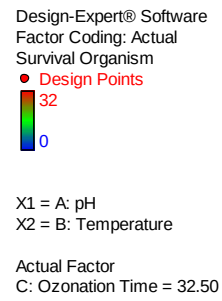
Fig 1. Normal Probability plot of actual and predicted values

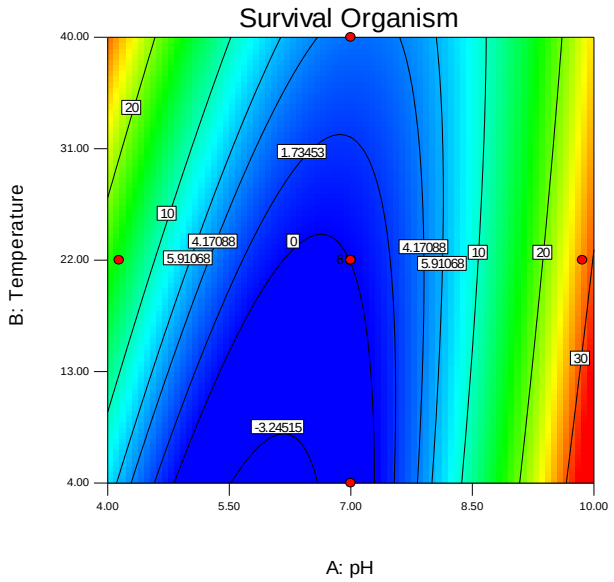
B. Effect of pH

The pH and type of acid play a major role in the inactivation of bacteria. Two inhibitory mechanisms involved in inactivation process are an intracellular acidification process (loss of homeostasis) and a specific effect of the acid (non-dissociated form) on metabolic activities. Inactivation efficiency of *L. monocytogenes* to specific pH (X_1) was studied by varying the pH from 4 to 10 of the phosphate buffered saline mixed with the same. The results obtained are shown in Fig. 2 and 3. From the figure it was observed that at a given temperature and time, increasing pH from acidic to neutral shows increasing inactivation. After neutral towards alkaline pH, efficiency of inactivation decreases. The pH between 4.5 and 7.5 shows the optimum range for the inactivation. *L. monocytogenes* maintains its intracellular pH within a narrow range of 7.6–8.0 and an extracellular pH value of 4.0 to 8.0 [28]. In this study it was observed that in similar conditions, at varying pH from 4 to 5, *L. monocytogenes* survival was around 30 CFU/mL to 3 CFU/mL. Whereas when the pH was increased gradually

from 5.5 to 7.5 there was a complete inactivation. An increase in pH from 8 to 10 the CFU/mL increased from 2 to 28. In this study it was found that low intracellular pH was not the major factor in the inhibition of *L. monocytogenes*. In fact, cells treated with organic acids or HCl at pH values as low as 3.5 were able to maintain their cytoplasmic pH near 5.0 [29].

The organism can become highly resistant to even extremely acidic conditions due to stress hardening [30]. Applying a mild acid stress actually increased the ozone treatment time required for a 5 log reduction of *L. monocytogenes* by comparison with the control population. It has been reported that pH, heat and acid resistance of *L. monocytogenes* are strain dependant [31-32].





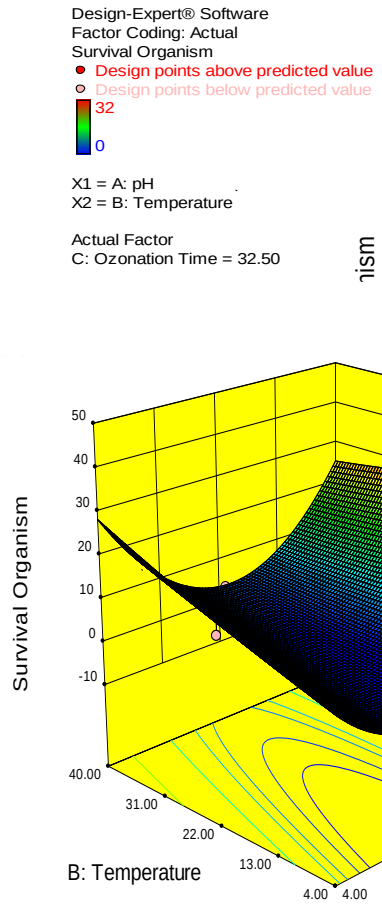
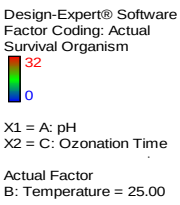


Fig. 2 Effect of pH and temperature on inactivation of *Listeria monocytogenes*

Fig. 3 Effect of pH and Ozonation time on the inactivation of *Listeria monocytogenes*



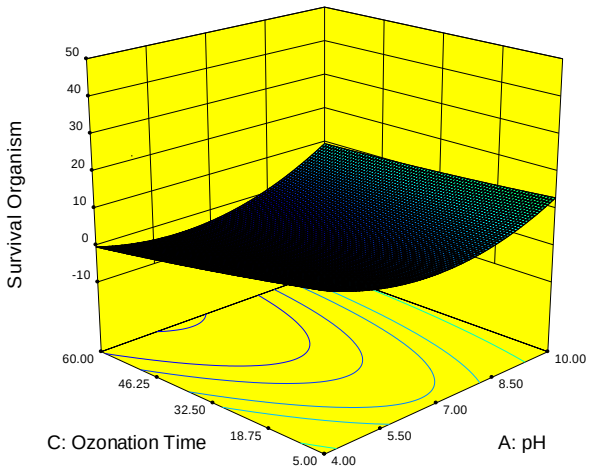


Figure 4 Effect of Temperature and Ozonation time on the survival of *Listeria monocytogenes*

C.Effect of ozonation time

To find the effect of ozonation time on the inactivation efficiency of *L. monocytogenes* experiments were conducted by varying the ozonation time (X_3) from 5 to 60 seconds. The results achieved are shown in FIG 3 and 4. The results revealed that increase in time increased the efficiency of ozonation. In this study, 33 seconds to 49 second exposure to ozone successfully inactivated 10^8 CFU/ml of *L. monocytogenes* and achieved a 7 log reduction. According to an ozonation study conducted using a batch reactor, conducted by, for the inactivation of *L. monocytogenes*. The treatment was most effective during the first 15 or 30 seconds while the counts of the organisms continued the similarity when the organism was exposed to the same dose till 6 min [39]. Whereas in a continuous reactor study conducted by the same authors most of the decrease in count occurred during the first 5 seconds of the treatment and the count of *L. monocytogenes* decreased gradually as contact time increased this kinetic pattern is more distinctive than that seen in other bacteria. Effective inactivations of L. monocytogenes to ozone exposure than other pathogenic microorganisms were well studied by Restaino et al., [40] who reported that even in the presence or absence of organic material, *L. monocytogenes* was more sensitive to ozonated water than the other Gram-positive and Gram-negative bacteria. This study produced unique results of inactivating the Gram-positive *L. monocytogenes* exposed less than 60 seconds where many authors have obtained the same results by exposing more than a minute [33]

IV. CONCLUSION

In conclusion, *L. monocytogenes* at an initial population of 8 log CFU/mL were inactivated using ozone. Three variables such as pH, temperature and ozonation time responsible for the inactivation of *L. monocytogenes* were optimized using response surface methodology. The model obtained has been validated and is expected to give reliable predictions in the specified ranges. It should be understood that the results used for developing this model is specific to the *L. monocytogenes* strain used in this study that can further be extended to other strains as well.

CONFLICT OF INTEREST STATEMENT

The author declare that there is no conflict of interest

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