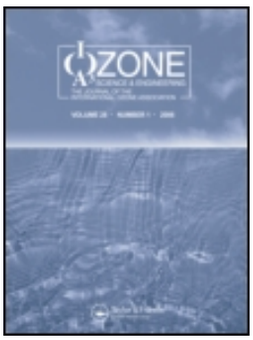

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Inactivation of *Bacillus cereus* and *Salmonella enterica serovar typhimurium* by Aqueous Ozone: Modeling and UV-Vis Spectroscopic Analysis

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ABSTRACT

Antibacterial efficacy of aqueous ozone (O_3) against *B. cereus* vegetative cells and *S. typhimurium* by was studied by using GInaFiT tool and results were validated using UV-Vis spectroscopy. Ozone gas was generated using a domestic ozone generator. Buffer solutions containing known amounts ($\approx 10^8$–10^9 cfu/mL) of bacterial pathogens were treated with aqueous ozone (200 mg/hr or 0.1 mg/l) for 16 min with sampling at 0-, 0.5-, 1-, 2-, 4-, 8- and 16-min intervals. A reduction of 4.6 log of *B. cereus* and 7.7 log cycle reduction of *S. typhimurium* was obtained in 16 min. Biphasic shoulder and double Weibull models were good fit for the experimental inactivation kinetics data. Principal Component Analysis showed discrete grouping based on the time of treatment. The highest correct classification results for SIMCA were achieved for both *B. cereus* and *S. typhimurium* after 1 and 8 min of treatment, respectively. In partial least squares regression analysis, maximum R^2 values for calibration and validation were found to be 0.84, 0.80 for *B. cereus* and 0.90, 0.89 for *S. typhimurium*, respectively.

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Bacillus cereus; Biphasic Model; Double Weibull Model; Kinetic Modeling; Ozone Treatment; Principal Component Analysis; *Salmonella typhimurium*

Introduction

Ozone (O_3) is a naturally occurring, pale blue gas. It is a triatomic allotrope of oxygen and is characterized by a high oxidation potential that confers bactericidal and viricidal properties (Burleson, Murray, and Pollard 1975; Kim, Yousef, and Dave 1999). FDA's approval of ozone as a direct additive to food in 2001 triggered interest in ozone applications. Its preservation efficacy has been evaluated in a variety of food products, including milk, gelatin, albumin, casein, and meat products (Kim, Yousef, and Dave 1999). The antibacterial activity of ozone has been attributed to its diffusion capability (Hunt and Marinas 1997). It reacts up to 3000 times faster than chlorine with organic material and it readily diffuses through biological cell membranes. Ozone inactivates microorganisms through oxidization and residual ozone decomposes to nontoxic products (oxygen) making it an environmentally friendly antimicrobial agent for use in the food industry.

Generally, the classical concept of log linear inactivation modeling fails to assess accurately the majority of survival curves obtained during ozone treatment. To overcome this problem, utilization of the time for four decimal reductions became widespread (Buchanan, Golden, and Phillips 1997; Membre, Thurette, and Catteau 1997; Whiting 1993). The concept of the time for four decimal reductions has the advantage of reflecting the evolution of the inactivation rate with respect to the various physicochemical factors studied, regardless of the patterns of various curves which can be related to a similar strain. GInaFiT is a user-friendly interface enabling easy identification of these curves on experimental data generated.

Ultraviolet and visible (UV-Vis) spectroscopy is a quantitative, reliable, and rapid analytical tool that can be immediately applied as a biosensor for the detection, identification, and enumeration of cells (Alupoaei et al. 2004; An and Friedman 1997). Multiwavelength transmission spectra of microorganisms and cell suspensions consist of combined absorption and scattering phenomena resulting from interaction of light with microorganisms or the cells typically suspended in a nonabsorbing media. The spectral absorbance properties are functions of the chemical composition and the state of aggregation, or association of the chromophoric groups contained in the microorganisms. UV-Vis spectroscopy of cells has been used to estimate the number

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of cells in a solution and their chemical composition, including nucleic acid and protein concentrations (Alupoaei et al. 2004; Leštan, Podgornik, and Perdih 1993). Based on the current uses of UV-Vis spectroscopy, it is feasible to develop a detection scheme to estimate the viability or survival of bacteria subjected a lethal treatment. Park et al. (2012) evaluated a method for the assessment of bacterial viability that is based on ultraviolet and visible (UV-Vis) spectroscopy analysis in the range of 200–290 nm. To our knowledge, there are no studies reported on application of spectroscopic and multivariance analysis to study the bacterial inactivation kinetics during ozone treatment.

Therefore, inactivation kinetics of *B. cereus* vegetative cells and *S. typhimurium* by aqueous ozone was studied by using GInaFiT tool and results were validated using UV-Vis spectroscopy.

Materials and methods

Bacterial strains and cultural conditions

Two bacterial species viz., *S. typhimurium* (MTCC No. 3224) and *B. cereus* (MTCC No. 1306) vegetative bacteria obtained from the microbial type culture collection and gene bank, Institute of Microbial Technology, Chandigarh (India) were used for inactivation studies. The freeze dried bacterial pellets were used for sub culturing in nutrient broth (Himedia Mumbai, India). A small amount of the revived cultures was plated onto nutrient agar yeast extract (NAYE; Himedia, Mumbai, India) for *S. typhimurium* and tryptic soy agar (TSA; Himedia, Mumbai, India) for *S. typhimurium* and incubated overnight at 30 °C and 37 °C, respectively. Thus obtained colonies were stored at 4 °C. Working cultures were prepared by inoculating a single colony into nutrient broth and incubating at optimal temperatures.

Preparation of vegetative bacterial cell suspensions

Cells grown in nutrient broth were harvested at stationary phase by centrifugation at 2300 *g* for 10 min at 4 °C. The cell pellet was washed twice with sterile phosphate buffer saline (PBS, Biorad, Bangalore, India). The pellet was resuspended in PBS and the bacterial density was determined by using the spread plate method. A working inoculum corresponding to approximately 1.0×10^{10} cfu/mL was prepared, and then serially diluted in 0.1% peptone water (Himedia, Mumbai, India) to obtain approximately 10^9 cfu/mL. Adding 10 ml of cell concentration (10^9cfu/ mL) to 90 ml of sterile PBS gave a final concentration of $\approx 10^8$cfu/mL.

Ozone treatment

Ozone gas was generated using a domestic ozone generator (Kent RO Systems Ltd., Noida, India). Ozone was produced by electrical discharge. Water and ambient air were the source of oxygen. The ozone output was 200 mg/hr. Ozone concentration was measured using redox potential (Younger 2010). Ozone concentration was also indirectly measured by measuring the absorbance spectra in the UV region of 200–250 nm. Experiments of inactivation of bacterial pathogens using aqueous ozone were conducted in a laminar flow. Sterile buffer solutions (PBS) containing known amounts of bacterial pathogens ($\approx 10^8$cfu cfu/mL) were treated for 16 min with sampling at 0-, 0.5-, 1-, 2-, 4-, 8- and 16-min intervals. All experiments were carried out in duplicate and replicated at least thrice.

Enumeration of surviving populations

The efficacy of treatments was determined in terms of reduction in viable counts over time. Populations of survivals of *B. cereus* and *S. typhimurium* were determined by plating onto NAYE and TSA, respectively. Samples (1 ml aliquots) were withdrawn from treated PBS at specific time intervals, serially diluted in 0.1% peptone water and 0.1 ml aliquots of appropriate dilutions were surface plated on NAYE and TSA to compare recovery of bacterial pathogens. Plates were incubated at 30 °C for *B. cereus* and 37°C for *S. typhimurium* for 24 h and then counted.

Statistical analysis

All experiments were repeated three times with duplicate samples and viable plate counts from three replications were reported as $\log_{10}$ cfu/mL. Data were pooled and average values and standard deviations were determined. Means were compared using ANOVA for completely randomized design followed by LSD testing at 5% significant level using the *T*-test.

Inactivation kinetics and modeling of survival curves

To describe the survival of *S. typhimurium* and *B. cereus*, different inactivation models available in the GInaFiT tool, namely the log-linear, log-linear +shoulder, Weibull, Double Weibull, biphasic and biphasic+ shoulder models (Van Boekel 2002) were

fitted to the data, and evaluated for goodness of fit. The GInaFiT tool was employed to perform the regression analysis of the microbial inactivation data (Geeraerd, Valdramidis, and Van Impe 2005). Inactivation kinetics parameters related to scale and shape of inactivation curves for most suitable models were calculated. The numerical values for inactivation kinetics parameters, time required to obtain 4-log reduction and other similar parameters were calculated for each model.

Spectroscopic evaluation of bacterial survivors after ozone treatment

The UV-Vis spectra of ozone treated bacterial samples were measured using a UV-Vis spectrophotometer (Shimadzu, Japan) at wavelengths ranging from 200 to 1100 nm with a 1-cm path length quartz cuvette. The scan speed and bandwidth of the spectrophotometer were 500 nm/min and 1.0 nm, respectively. Prior to recording the spectrum of each sample, the spectrophotometer was zeroed to account for any stray light. To avoid the effect of inhomogeneities in the suspending medium, the background spectrum was determined using the corresponding suspending media from the batch used to prepare the original sample. The temperature and humidity of the laboratory were 25 ± 1 °C and 20 ± 3% RH (relative humidity), respectively, when spectroscopic measurements were carried out.

Digital spectra and optical density in the range of 200–290 nm was determined to know the different proportion of bacterial survivals after ozone treatment. A correlation between number of bacteria and optical density was calculated. Further, multivariate data analysis was carried out using Unscrambler X software (Camo, India, Ltd.). Thus obtained spectra were analyzed using regression techniques, and classification methods like soft independent modeling for class analogy (SIMCA), Principal component analysis (PCA) was also carried out to disclose naturally occurring groups and structures within the data set without previous knowledge of class assignment. Partial least squares regression (PLSR) was used for prediction and quantification purposes.

Results and discussion

Measurement of ozone concentration

In this study, the measured ozone concentration was 0.1 ppm (0.1 mg/L). Ozone solution showed a peak absorbance at 210–230 nm. Ozone is a strong oxidizing agent that has a high oxidation potential. This measurement is called redox potential, which is an indirect measurement of ozone concentration in water (Younger 2010). In earlier study also, Daumont et al. (1992) observed the peak absorption spectrum of ozone in the UV region of 230–250 nm range.

Effect of ozone on inactivation of B. cereus *and* S. typhimurium *suspended in PBS*

Ozone inactivation of bacteria suspended in PBS was initiated after 0.5 min. An exposure to ozone (0.1 mg/L) for 16 min resulted in a 4.6 log cycle reduction of *B. cereus*. A similar treatment resulted in 7.7 log cycle reduction of *S. typhimurium*. Biphasic shoulder model and double Weibull model were able to describe food borne pathogens inactivation kinetics during ozone treatment. The parameters of biphasic shoulder model are shown in Table 1. Among two bacteria studied, k_{max} (maximum death rate specific inactivation) was significantly higher for *S. typhimurium*. The time required to obtain 4-log reduction (4-D value) was significantly ($p < 0.05$) higher in *B. cereus*. Analysis of kinetics data showed that biphasic shoulder model was a good fit for the experimental data obtained. Both bacteria studied were susceptible to ozone treatment, but experimental inactivation data and predicted parameters indicated that *B. cereus* is more resistant to ozone treatment than *S. typhimurium*.

The direct application of ozone was found to be effective for the inactivation of *B. cereus* and S. *typhimurium* but sensitivity to ozone varied between

Table 1. Parameters of the biphasic shoulder model and double weibull model and the time required to reach a 4 log reduction for *B. cereus* and *S. typhimurium*.

Bacterial species	K_{max1} (1/min)	K_{max2} (1/min)	Shoulder	4-D (min)	R^2	RMSE	
Biphasic Shoulder model parameters							
B. cereus	7.15 ± 0.54[a]	0.26 ± 0.05[a]	0.28 ± 0.05[a]	9.15 ± 0.60[a]	0.99 ± 0.0	0.24 ± 0.06	
S. typhimurium	10.75 ± 1.32[b]	1.41 ± 0.05[b]	0.56 ± 0.03[b]	3.51 ± 0.18[b]	0.99 ± 0.0	0.26 ± 0.03	
Bacterial species	α	δ1	p	δ2	4-D	R^2	RMSE
Double Weibull model parameters							
B. cereus	3.37 ± 0.12	0.44 ± 0.05	1.16 ± 0.12	7.12 ± 0.85[b]	9.31 ± 0.60[b]	0.95 ± 0.06	0.36 ± 0.40
S. typhimurium	3.11 ± 0.16	0.73 ± 0.04	1.51 ± 0.12	3.06 ± 0.26[a]	3.41 ± 0.14[a]	0.99 ± 0.01	0.31 ± 0.27

Means within a column with different superscripts differ significantly ($p < 0.05$).

the bacterial species. Recently Sengun (2013) reported that using ozonated wash water (0.5–1.5 ppm) in vegetables reduced *S. typhimurium* by 1.14 to 3.06 log units. Pascual, Liorca, and Canut (2007) suggested that the effectiveness of ozone against microorganisms depends not only on the amount applied, but also on the residual ozone in the medium, various environmental factors such as medium pH, temperature, humidity, additives (surfactants, sugars, etc.) and the amount of organic matter surrounding the cells. Ozone achieves inactivation of bacteria by having an effect on various cellular components like proteins, peptidoglycans in cell envelopes, enzymes and nucleic acids in the cytoplasm (Khadre, Yousef, and Kim 2001).

The Biphasic model identified for S. typhimurium is: $\log_{10}(N) = \log_{10}(N0) + \log_{10}(f(\exp(-k_{max1})t)) + (1\text{-}f)(\exp(-k_{max2})t))$.

For identification purposes, it was reformulated as: $\log_{10}(N) = \log_{10}(N0) + \log_{10}(f(\exp(-k_{max1})t) + (1\text{-}f)\exp(-k_{max2})t))$ (Cerf 1977).

The double Weibull parameters α, δ and p and 4-D values are shown in Table 1. The shape parameter p showed downward concavity for both bacteria (Figures 1 and 2).The inactivation kinetics data of *B. cereus* and *S. typhimurium* was fitted using the double Weibull model, which provided estimations of microbial inactivation in terms of processing time required. Results showed that show that the double Weibull model was also a good fit for the experimental data analyzed. The double Weibull parameters values like α, δ1 were not significantly different for both bacteria studied. However, δ2 and the 4-D values were significantly ($p < 0.05$) higher for *B. cereus* as compared to *S. typhimurium*. Estimated parameters also indicated the

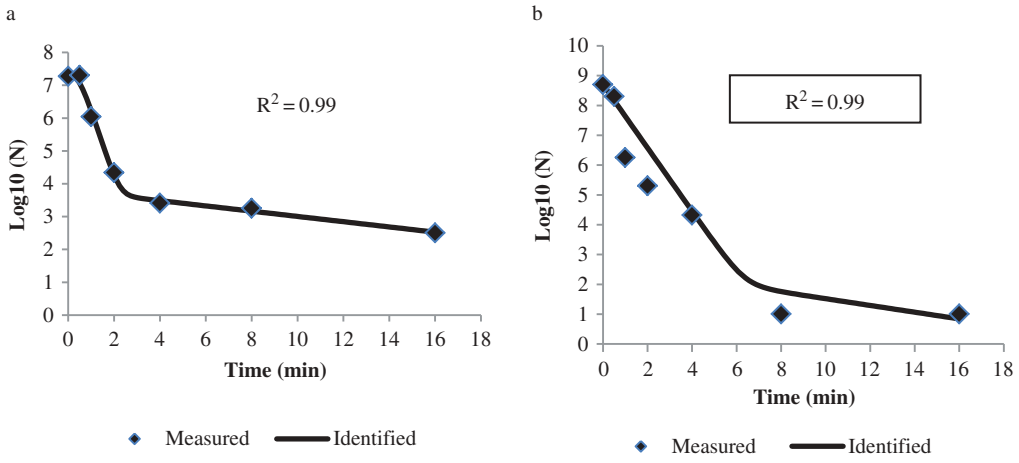
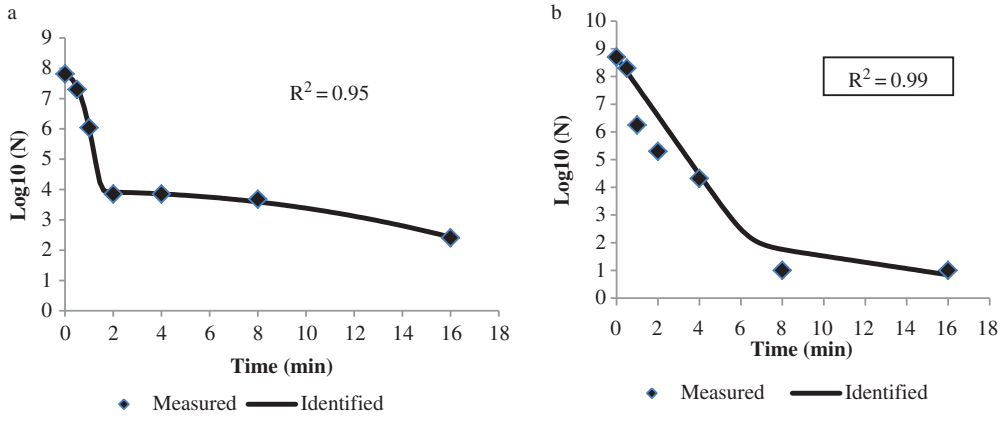


Figure 1. Microbial survival curve of *B. cereus* and *S. typhimurium* curves are fitted using the biphasic shoulder model. (1a. *B. cereus*. 1b. *S. typhimurium*).



Figures 2. Microbial survival curve of *B. cereus* and *S. typhimurium* curves are fitted using the double Weibull model. (1a. *B. cereus*. 1b. *S. typhimurium*).

higher sensitivity of *S. typhimurium* to ozone than *B. cereus*. For the double Weibull model, the inactivation model identified for both bacteria is:

$$N = N0/(1 + 10^{\wedge}\alpha)(10(-((t-1)/\delta 1)^{\wedge}p + \alpha) + 10^{\wedge}(-((-1)/d\delta 2)(p)))$$

For identification purposes, it was reformulated as: Log_{10} (N) = log_{10} (10) N0/ (1 + 10) α) (10 (-(t/δ1) p + α) +10 (-(t/δ2) p))) (Coroller et al. 2006). Where, *N*0 is the initial size of the population; δ is the time of the first logarithm decline for the two subpopulations; α is defined as the logit of *f* and is equivalent to log_{10} (*N*01/*N*02), and *p* represents the shape of the curve.

In biphasic model, initial population is divided into major (susceptible) and minor (resistant) subpopulations and k_{max1} and k_{max2} (1/time unit) are the specific inactivation rates of the two populations, respectively. This biphasic model can be written under the form of two first-order differential equations, one for N1 (the major subpopulation) and one for N2 (the minor subpopulation). Biphasic inactivation curves have been observed in the framework of thermal inactivation (Cerf 1977; Humpheson et al. 1998) and nonthermal inactivation due to lethal water activity or lethal pH levels (Shadbolt, Ross, and McMeekin 2001). Survivor curves of biphasic with a preceding shoulder are rather uncommon. Whiting (1993) modeled the survival of *L. monocytogenes* and three *Salmonella* strains in BHI broth under stressful conditions and observed a similar curve. In this model, the major population is more sensitive (k_{max1} is larger than k_{max2}). Furthermore, the concept of the time for four decimal reductions has the advantage of reflecting the evolution of the inactivation rate with respect to the various physicochemical factors regardless of the patterns of various curves, which can be related to a similar strain.

The Weibull and double Weibull model have largely been used in thermal and nonthermal treatment studies. It is based on the hypothesis that the resistance to stress of a population follows a Weibull distribution (Corradini and Peleg 2003; Hajmeer et al. 2006; Van Boekel 2002). The evolution of the parameter values related to models was directly linked to the increase in the resistance. The double Weibull simplified model has five parameters, but only three of these parameters (δ1, δ2, and α) are related to the physiological state of the cells and environmental conditions. The double Weibull simplified model is more flexible and could describe the biphasic nonlinear shape (*p* greater than 1), as well as the biphasic linear case (*p* equal to 1).

UV-Vis Spectroscopic Analysis

The spectra of optical density for of *B. cereus* and *S. typhimurium* subjected to different ozone treatment times are shown in Figures 3 and 4 respectively. Each spectrum represents an average of 10 repeated measurements. A similar spectral absorption behavior was observed for *B. cereus and S. typhimurium*. UV-Vis spectral study results indicated that the optical density in the range of 200 nm to 1100 nm decreased as the level of inactivation increased. The maximum difference between spectra of different samples was observed in the wavelength range of 230–320 nm. But that the

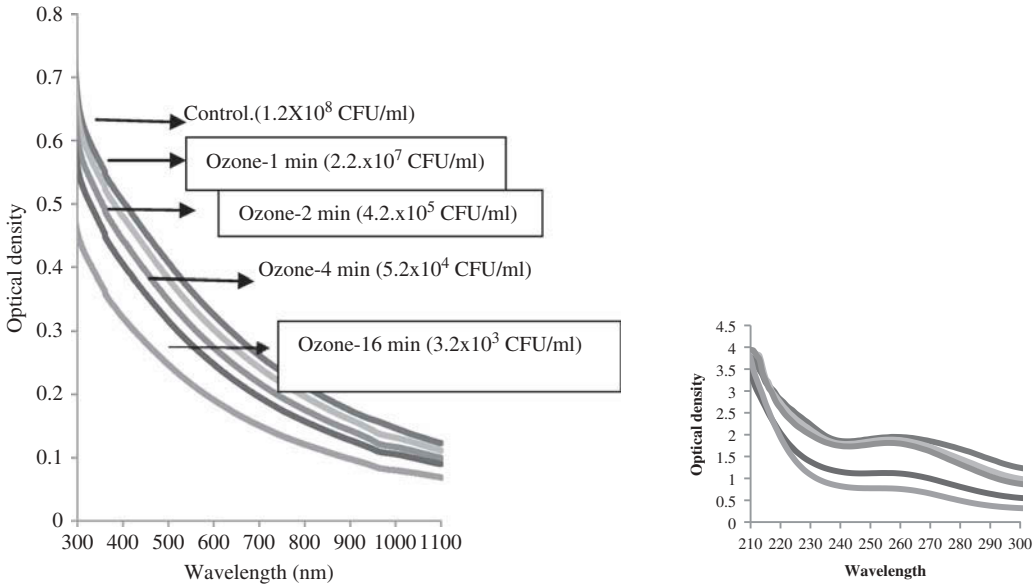


Figure 3. Spectra of normalized optical density versus the number of survival *B. cereus* bacteria after ozone treatment.

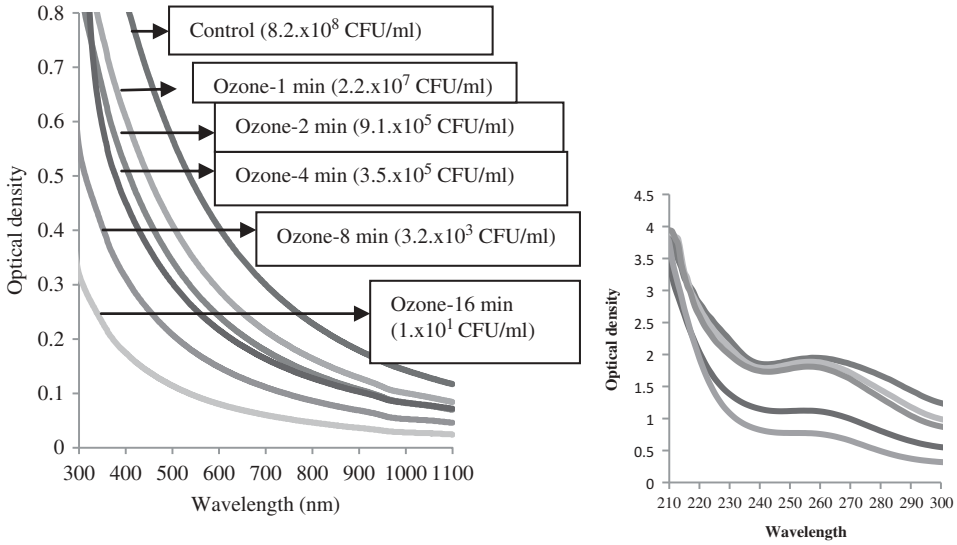


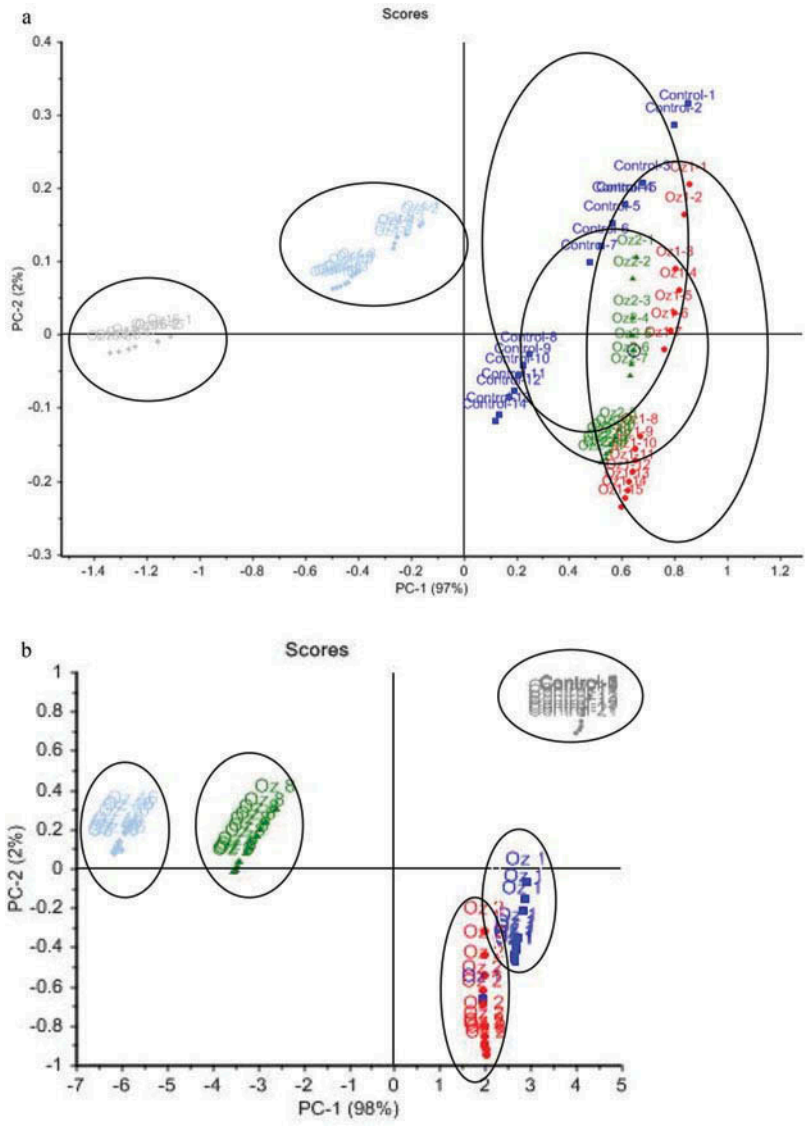
Figure 4. Spectra of normalized optical density versus the number of survival bacteria *S. typhimurium* after ozone treatment.

optical density measured in the wavelength range longer than approximately 700 nm did not correlate well with the level of inactivation of live bacteria. Spectral absorbance at 230 nm (Figures 3 and 4) were generally attributed to the absorption of DNA (one of the intracellular materials); therefore, in this study, the amplitude of the peak at 230 nm was influenced by the level of inactivation or in other words number of survival and inactivated bacterial cells.

Further, PCA, used in this study as a data reduction tool was found to be useful in classification as the graphical display of scores discloses clustering patterns. The score plot following PCA of the bacterial (*B. cereus*) treated with ozone for different time interval showed discrete grouping based on the time of treatment. The sum of variances described by PC 1 and 2 was 99% at 5% significance level (Figure 5a). This indicated that treatment time had significant effect on the bacterial viability (*B. cereus.*). Five groups were formed based on time of treatment with ozone (Figure 5). This indicated that classification of samples based on time of treatment with ozone, which in turn caused death of cells are possible, with a significance level of 5%. In other words, classification of samples based on percentage of live and dead cells is possible. Similar studies were conducted with *S. typhimurium*. Results showed that the sum of variances described by PC 1 and 2 was 100% at 5% significance level (Figure 5b). Again five clear clusters were observed based on the treatment time.

After ozone treatment the surviving population was enumerated based on percentage of live: dead bacteria, different class models were built and test samples (for both bacteria) were classified using SIMCA approach. A summary of model performance for *B. cereus* and *S. typhimurium* is shown in Tables 2 and 3, respectively. The highest correct classification results for SIMCA were achieved without any transformation of spectral data in the range of 230–320 nm for *B. cereus* (Table 2). However, in the case of *S. typhimurium* correct classification was obtained when the bacterial culture was treated with ozone for 8 or 16 min, which resulted in death of 89% of the population. Although the culture treated with ozone for 2 min showed misclassification in neighboring class. The control cultures were also misclassified in the class of 1 minute treated population. Discriminating power of SIMCA is a measure of variable importance in spectral frequency and contributes to the development of the classification model (Dunn and Wold 1995).

Partial least squares regression (PLSR) was used for prediction and quantification purposes. It is linear regression method that forms components or factors as new independent explanatory variables in a regression model. PLS with the option of full cross-validation were performed on selected spectral window (230–320 nm) using calibration set of samples for predicting percentage of live: dead bacteria. The spectral windows were selected based on the maximum visible differences in peaks and depression in the spectra of bacterial cultures treated with ozone for different time interval. The best models based on the standard error of calibration (SEC), coefficient of determination (R^2) and standard error of prediction (SEP) were selected (Jaiswal, Jha, and Bharadwaj 2012) for quantification of percentage of live: dead bacteria.



Figures 5. UV-Vis spectral analysis of control and ozone-treated samples: a. Principal component analysis of UV-Vis spectra of *B. cereus*. b. Principal component analysis of UV-Vis spectra of *S. typhimurium*.

Table 2. SIMCA classification results for percentage of live: dead bacteria (*B. cereus*) after ozone treatment.

Wavelength range	Duration of ozone treatment (live+dead)	No. of PCs	No. of test samples	Number of sample classified					Correct classification (%)
				100% Live	78% live	63% live	60% live	33% live	
230-320nm	0 min (100% Live)	7	6	6					
	1 min (78% Live)		8		8				
	2 min (63% Live)		7			7			
	4 min (60% Live)		8				8		
	16 min (33% Live)		8					8	

Maximum R^2 values for calibration and validation were found to be 0.84 and 0.80, respectively, in the range of 230–320 nm using PLS for *B. cereus*. The R^2 values for calibration and validation in case of *S. typhimurium* was found to be 0.89 and 0.88, respectively. Scatter plots of actual vs. predicted values obtained from the model for detection of percentage live bacteria after treatment with ozone were plotted to have an idea of predictability. The slope of 45° with negligible and distribution of sample on scatter plots between observed and predicted values (Figures 6 and 7) showed that prediction is close to the experimental values.

Table 3. SIMCA classification results for percentage of live: dead bacteria (*S. typhimurium*) after ozone treatment.

Wavelength range	Duration of ozone treatment (live+dead)	No. of PCs	No. of test samples	Number of sample classified					Correct classification (%)
				100% Live	82% live	62% live	18% live	11% live	
230-320 nm	0 min (100% Live)	7	10	10	10				
	1 min 82% Live		11		10				
	2 min 62% Live		10		4	8			
	8 min 18% Live		10				10		
	16 min 11% Live		11					10	

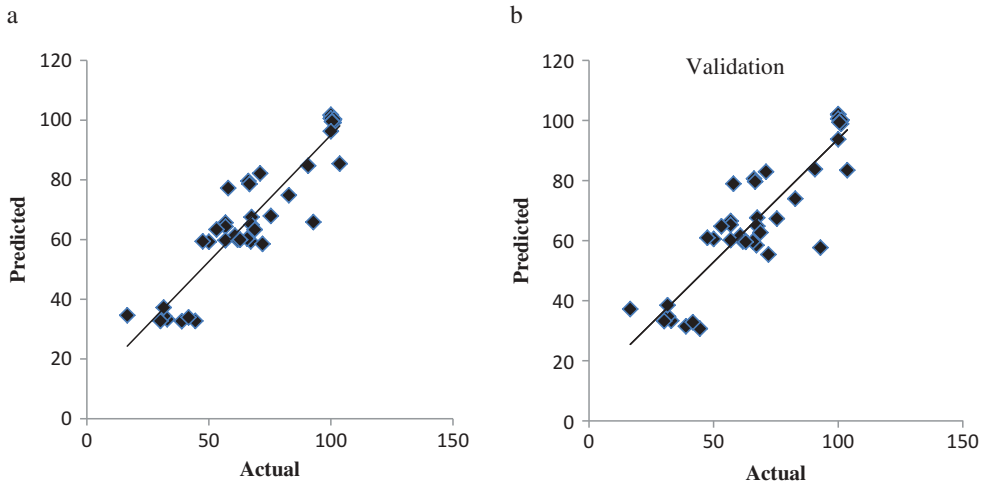


Figure 6. Observed vs. predicted levels (%) of live and dead cells of *B. cereus* using PLS in the range of 230–320 nm for calibration (a) and validation (b) sets of samples.

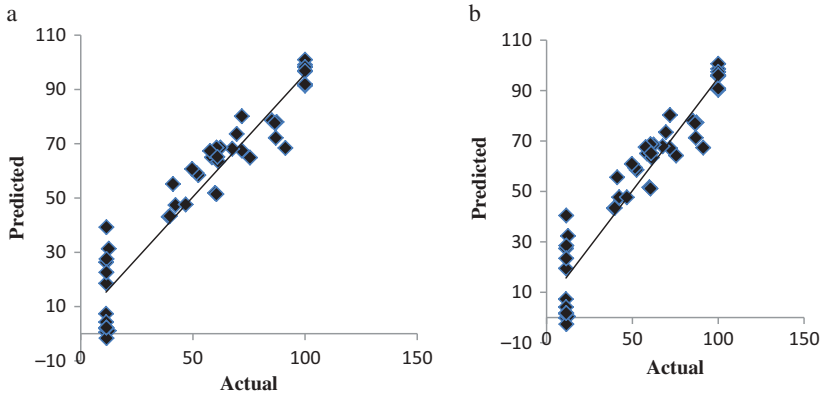


Figure 7. Observed vs. predicted levels (%) of live and dead cells of *S. typhimurium*. using PLS in the range of 230–320 nm for calibration (a) and validation (b) sets of samples.

Conclusion

The efficacy of ozone treatment was found to be a function of treatment time. Inactivation times for a 4 log cycle reduction ranged between 9.14 and 4.51 min for *B. cereus* and *S. typhimurium*, respectively. UV-Vis spectroscopy and chemometric analysis showed that inactivation kinetics can be described using PCA, SIMCA and PLS. Further UV-Vis spectral analysis could able to differentiate the percentage of live and dead bacteria after ozone treatment.

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