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Extrinsic Control Parameters for Ozone Inactivation of Escherichia Coli Using a Bubble Column

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ORIGINAL ARTICLE

Extrinsic control parameters for ozone inactivation of *Escherichia coli* using a bubble column

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Keywordsbubble column, *E. coli*, nonthermal technology, ozone, Weibull model.**Correspondence**

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Abstract**Aims:** To investigate the effect of extrinsic control parameters for ozone inactivation of *E. coli* in a bubble column.**Methods and Results:** Ozone inactivation of *Escherichia coli* ATCC 25922 in Tryptic Soya Broth was examined. The parameters studied included temperature (ambient, 20, 25 and 30°C), exposure time (up to 30 min), gas flow rate (0.03, 0.06, 0.12, 0.25, 0.5 and 0.75 l min⁻¹) and concentration level (five different levels). The efficacy of ozone treatment was a function of the parameters investigated and optimum control parameters of flow rate (0.12 l min⁻¹), temperature (ambient) and ozone concentration (75 µg ml⁻¹) resulted in a *t*₄₅ (time required to achieve 5 log reduction) of 20 min.**Conclusions:** Optimum control parameters of gas flow rate, ozone concentration and temperature are reported for *E. coli* inactivation within a bubble column.**Significance and Impact of the Study:** In 2001, the FDA approved use of ozone as a direct additive to food and in 2004, issued guidelines for the use of ozone in liquid systems. However, these guidelines highlighted gaps in the literature for ozonation of liquid foods. This study provides useful information regarding optimum extrinsic control parameters for *E. coli* inactivation in liquid media using a bubble column to ensure microbiological safety.**Introduction**

Ozonation is a relatively new method for food preservation which can be applied in order to meet consumer's demand for fresher and safer ready-to-eat products. Ozone destroys micro-organisms by progressive oxidation of vital cellular components. Activated oxygen species resulting from ozone decomposition includes singlet oxygen, hydroxyl radical, superoxide anion (perhydroxyl radical at low pH) and hydrogen peroxide which elicit potent cidal activity against a broad-spectrum of micro-organisms (Korycka-Dahl and Richardson 1978). The bacterial cell surface has been suggested as the primary target of ozonation. The biocidal effect of ozone is caused by its high oxidation potential, reacting up to 3000 times faster than chlorine with organic material (EPRI 1997), and its ability to readily diffuse through biological cell membranes. Khadre and Yousef (2001) found that ozone

was more effective than hydrogen peroxide against food borne *Bacillus* species spores. Treatments with ozone (1.6 and 2.2 ppm) for 1 min decreased *Shigella sonnei* population in water by 3.7 and 5.6 log, respectively. In addition, *S. sonnei* counts were reduced by 1.8 log units in lettuce treated with 5 ppm for 5 min (Selma *et al.* 2007). Oztekin *et al.* (2005) reported on the effects of ozone treatment on the micro flora of dried figs, where the application of gaseous ozone at 5 or 10 ppm for 3–5 h resulted in significant reductions in total bacteria, coliform and yeast/mould counts. Habibi Najafi and Haddad Khodaparast (2009) concluded that a minimum of one hour ozone treatment at 5 ppm could be successfully used for reducing both coliform and *Staphylococcus aureus* populations on date fruits, but that longer exposure times are required for elimination of the total mesophilic bacteria as well as yeast/mould. Fan *et al.* (2007) reported that gaseous ozone effectively inactivated *Listeria innocua* on solid

media at concentrations of 50 and 100 nl l^{-1} during short exposure times at both 5 and 20°C. Ozone has been shown to reduce populations of *E. coli* O157:H7 in phosphate buffer (Byun *et al.* 1998) while its preservation efficacy has been also evaluated in a variety of food products, including milk, gelatin, albumin, casein, and meat products (Kim *et al.* 1999). Williams *et al.* (2004) reported that ozone treatment (0.9 g h^{-1}) of apple cider and orange juice at 4°C or ozone in combination with mild heating (50°C) may provide an alternative to thermal pasteurization for reduction of *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice. The major advantage of ozone is auto decomposition, excess ozone auto-decomposes rapidly to produce oxygen thus leaving no residues in food (Khadre *et al.* 2001). Ozone is readily soluble in water and its solubility increases as the temperature of water decreases (Steenstrup and Floros 2004). *Escherichia coli* O157:H7 is an enteric pathogen that is associated with a number of outbreaks of food-borne illnesses (CDC 1996). These outbreaks led the U.S. Food and Drug Administration (FDA) to issue hazard analysis critical control point (HACCP) regulations for safe and sanitary processing of liquid foods such as fruit juice (USFDA 2001). A primary performance standard required by this HACCP regulation is a minimum 5-log reduction in the juice being processed (USFDA 2001).

The FDA's approval of ozone as a direct additive to food in 2001 triggered interest in ozone applications. A number of commercial fruit juice processors in the US and Europe began employing ozone to treat the products resulting in industry guidelines being issued by the FDA (2004). However, these guidelines highlighted gaps with respect to lack of knowledge regarding the critical control parameters for ozone inactivation in liquid systems. The objective of this study was to investigate the effect of extrinsic control parameters for ozone inactivation of *E. coli*.

Materials and methods

Bacterial culture conditions

Stock cultures of *E. coli* ATCC 25922 were maintained using protect beads (Technical Services Consultants Ltd, Lancashire, UK) at -70°C . One protect bead was used to inoculate one tryptic soy agar (TSA; Scharlau Chemie, Barcelona, Spain) plate, and incubated at 37°C overnight. An isolated colony of *E. coli* ATCC 25922 was inoculated into 5 ml tubes containing tryptic soy broth (TSB) and incubated overnight at 37°C. The bacterial density was determined by measuring absorbance at 550 nm using McFarland standard (BioMérieux, Marcy-l'Étoile, France) working inoculum corresponding to $1.0 \times 10^8 \text{ CFU ml}^{-1}$

was prepared. For ozone treatment, cells were adjusted to a density of $1.0 \times 10^6 \text{ CFU ml}^{-1}$ in TSB.

Ozone generation and measurements

Ozone was produced by a corona discharge generator (Model OL80, OzoneLab™; Ozone Services, Burton, Canada). Pure oxygen was supplied via an oxygen cylinder (Air Products Ltd, Dublin, Ireland) and the flow rate was controlled using an oxygen flow regulator. To determine the effect of gas flow rate and ozone concentration, experiments were carried out in a 100 ml glass bubble column. In order to determine the effect of temperature, experiments were carried out in a 250 ml glass bubble column with heating jacket (Fig. 1). Ozone concentration was varied from 28–120 $\mu\text{g ml}^{-1}$ (Table 1). Ozone concentration was recorded using an ozone analyzer. Temperature was controlled in the 250 ml bubble column by

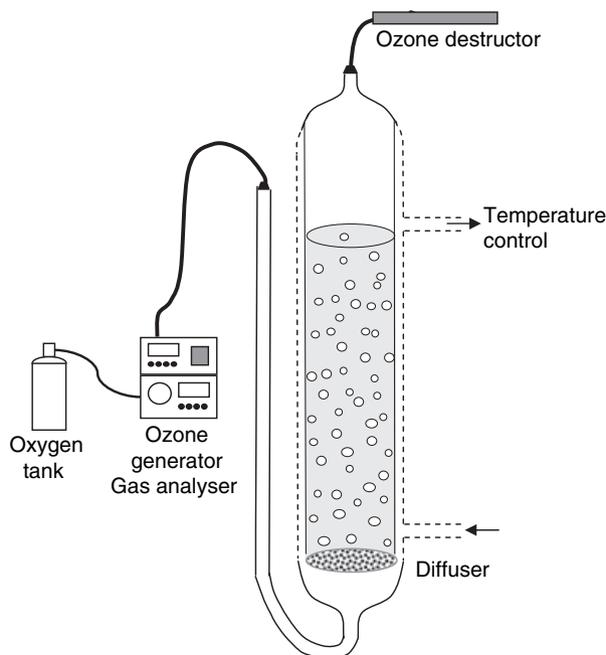


Figure 1 Schematics of the ozone processing equipment.

Table 1 Ozone concentrations at two selected gas flow rates

Levels of ozone concentration	Flow rate 0.06 l min^{-1} concentration ($\mu\text{g ml}^{-1}$)	Flow rate 0.12 l min^{-1} concentration ($\mu\text{g ml}^{-1}$)
Level 1	28–49	17–28
Level 2	60–70	33–40
Level 3	82–87	46–56
Level 4	103–114	67–70
Level 5	115–120	72–75

circulating water at the selected temperature through the heating jacket. Steady state ozone production was achieved prior to media treatment by passing ozone through sterile distilled water for 10 min at the required flow rate and temperature. To prevent excess foaming, 5–10 μl sterile anti-foaming agent (Sigma Aldrich Ireland Ltd, Dublin, Ireland) was added to the medium before each ozone treatment.

Experimental design

A working concentration of 1×10^6 CFU ml^{-1} *E. coli* ATCC 25922 inoculum was treated at gas flow rates of 0.03, 0.06, 0.12, 0.25, 0.5 and 0.75 l min^{-1} . Inoculated samples of 90 ml for the 100 ml column or 200 ml for the 250 ml column were transferred to the bubble column reactors, and treated for up to 30 min. Experiments were also carried out to determine the effect of ozone concentration and temperature on the efficacy of ozone for inactivation of 1×10^6 CFU ml^{-1} *E. coli* ATCC 25922. Different ozone concentrations in the range of 28–120 and 17–75 $\mu\text{g ml}^{-1}$ were tested. For the temperature studies, different temperatures (12–15°C, 20°C, 25°C and 30°C) were tested at optimum flow rate of 0.12 l min^{-1} achieved on the basis of flow rate studies. All experiments were carried out in duplicate and then replicated.

To quantify the effects of ozone treatment parameters, 1 ml samples were removed at 5 min intervals; samples were serially diluted in maximum recovery diluent (MRD; Scharlau Chemie), and 0.1 ml aliquots of appropriate dilutions were plated on TSA in duplicate, incubated at 37°C for 24 h and counted. Control experiments were performed where the working inoculum was exposed to oxygen only.

Mathematical modelling

Primary modelling

The GInaFiT tool was employed to perform the regression analysis of the microbial inactivation data (Geeraerd *et al.* 2005). The Weibull model (Mafart *et al.* 2002) was used to model the experimental death curves, which displayed a downward concave trend. This model has been used previously to describe the inactivation kinetics for ozone (Bialka *et al.* 2008). The model used was:

$$\text{Log}_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\alpha}\right)^\beta \quad (1)$$

where N is the number of micro-organisms, N_0 (CFU ml^{-1}) is the initial number of micro-organisms, α [min] (time for the first decimal reduction) and β are parameters relating to the scale and shape of the inactivation curve, respectively. The numerical values of α

and β were used to calculate a desired log reduction. The time required to obtain a 5 log reduction (t_{d5}) was calculated using eqn (2), where D_5 is 5 log reduction

$$t_{d5} = \alpha \times (D_5)^{\frac{1}{\beta}} \quad (2)$$

The Weibull distribution corresponds to a concave upward survival curve if $\beta < 1$ and concave downward if $\beta > 1$ (van Boekel 2002).

Secondary modelling

To characterize the dependence of the Weibull parameters (α and β) on the ozone processing variables, the following steps were taken:

1. Individual nonlinear regressions of all experiments with eqn (1) were performed.
2. Inspection of the variation of α and β with processing temperature (T) and ozone flow rate per reactor volume ($f\text{O}_3$) ($\text{mg min}^{-1} \text{ml}^{-1}$).
3. Experiments at each processing condition were averaged.
4. Polynomial relationships of $\ln(\alpha)$ and β with the ozone flow rate and the temperature were proposed.
5. Additional terms to the model were investigated and compared with the base model using a log-likelihood ratio test.

All nonlinear regressions were performed using the *R* statistical software libraries (*R* Core Development Team, 2007).

Results

Effect of gas flow rate

The inactivation of *E. coli* ATCC 25922 was found to be dependant on gas flow rate. Survival curves of *E. coli* ATCC 25922 in TSB treated with ozone (0.045–0.170 $\text{mg min}^{-1} \text{ml}^{-1}$) at the selected flow rates are shown in Fig. 2. Each curve shows a noticeable lag time prior to reduction, due to ozone demanding substances present in the TSB medium. Regardless of the lag time, after 25 min, complete inactivation was achieved using the following flow rates: 0.06, 0.12, 0.25 and 0.5 l min^{-1} . However, complete inactivation was not achieved with flow rates of 0.03 and 0.75 l min^{-1} after 30 min treatment. The optimum flow rate was 0.12 l min^{-1} , with a t_{d5} value of 20 min (Table 2). Exposure of *E. coli* to pure oxygen for 30 min resulted in no inactivation (data not shown).

Effect of ozone concentration

The optimum flow rate of 0.12 l min^{-1} was chosen to investigate the effect of ozone concentration on

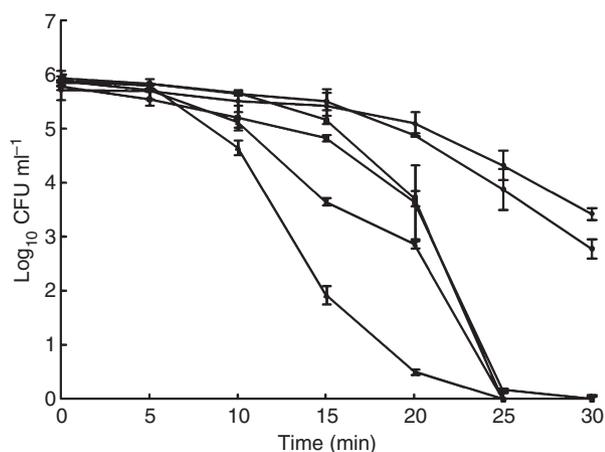


Figure 2 Ozone inactivation of *E. coli* at different flow rates. Flow rates: (◆) 0.03, (■) 0.06, (▲) 0.12, (×) 0.25, (□) 0.5, (●) 0.75. Error bars represent SD.

inactivation. Based on results reported by Tiwari *et al.* (2008a), a second flow rate of 0.06 l min⁻¹ was investigated as this flow rate was found to incur the least amount of colour degradation for freshly squeezed orange

juice. Five different levels of ozone concentration were investigated; ranging from 28 to 120 µg ml⁻¹ ozone in the case of 0.06 l min⁻¹, and from 17 to 75 µg ml⁻¹ ozone in the case of 0.12 l min⁻¹ (Fig. 3 and Table 1). For both flow rates, the highest concentration (level 5) was the most effective to inactivate *E. coli* ATCC 25922 (Fig. 3a,b). The flow rate of 0.06 l min⁻¹ yielded a t_{d5} of 24.2 min and the 0.12 l min⁻¹ flow rate yielded a t_{d5} value of 20 min. From the graph it is evident that there is a stepwise decrease in the efficiency of *E. coli* inactivation at the lower concentration levels (1 and 2), where inactivation was insufficient to calculate t_{d5} values.

Effect of temperature

Temperature effects on the efficacy of ozone inactivation on *E. coli* ATCC 25922 were examined at the optimum inactivation flow rate of 0.12 l min⁻¹. Four different temperatures were investigated: ambient temperature (12–15°C), 20°C, 25°C and 30°C. Survival curves are shown in Fig. 4. Ambient temperature gave the best inactivation levels with a t_{d5} value of 20 min (Table 2).

Table 2 Parameters of the Weibull model and extrinsic control parameters for ozone inactivation of *E. coli*

Extrinsic parameters	fO ₃ (mg O ₃ min ⁻¹ ml ⁻¹)	α (min)	β	R ²	t _{d5} (min) inactivation time
Gas flow rate (l min ⁻¹)					
0.03	0.045	22.98 ± 0.51*	3.07 ± 0.23	0.99	38.82
0.06	0.078	16.88 ± 0.20	4.48 ± 0.13	0.99	24.2
0.12	0.098	5.53 ± 2.62	1.25 ± 0.4	0.95	20
0.25	0.145	11.65 ± 1.67	2.27 ± 0.4	0.98	23.7
0.5	0.144	16.60 ± 0.81	3.83 ± 0.44	0.99	29.5
0.75	0.170	15.31 ± 0.67	2.28 ± 0.19	0.99	31
Temperature (°C)					
Ambient	0.098	5.53 ± 2.62	1.25 ± 0.4	0.95	20
20	0.087	19.18 ± 0.93	4.08 ± 0.13	0.99	28.5
25	0.087	27.09 ± 0.37	4.22 ± 0.42	0.99	39.7
30	0.087	23.6 ± 0.45	7.46 ± 0.48	0.99	29.3
Concentration (levels)					
0.06 l min ⁻¹ flow rate					
1	0.025	–	–	–	–
2	0.043	–	–	–	–
3	0.056	25.68 ± 0.71	2.38 ± 0.38	0.98	50.54
4	0.072	22.40 ± 0.39	2.54 ± 0.22	0.99	42.2
5	0.078	16.88 ± 0.20	4.48 ± 0.13	0.99	24.18
Concentration (levels)					
0.12 l min ⁻¹ flow rate					
1	0.03	–	–	–	–
2	0.048	37.01 ± 4.28	1.52 ± 0.39	0.96	107.04
3	0.068	18.3 ± 0.61	3.70 ± 0.37	0.99	28.27
4	0.087	15.50 ± 0.34	3.51 ± 0.16	0.99	24.5
5	0.098	5.53 ± 0.62	1.25 ± 0.37	0.95	20

R², coefficient of determination.

*Standard error.

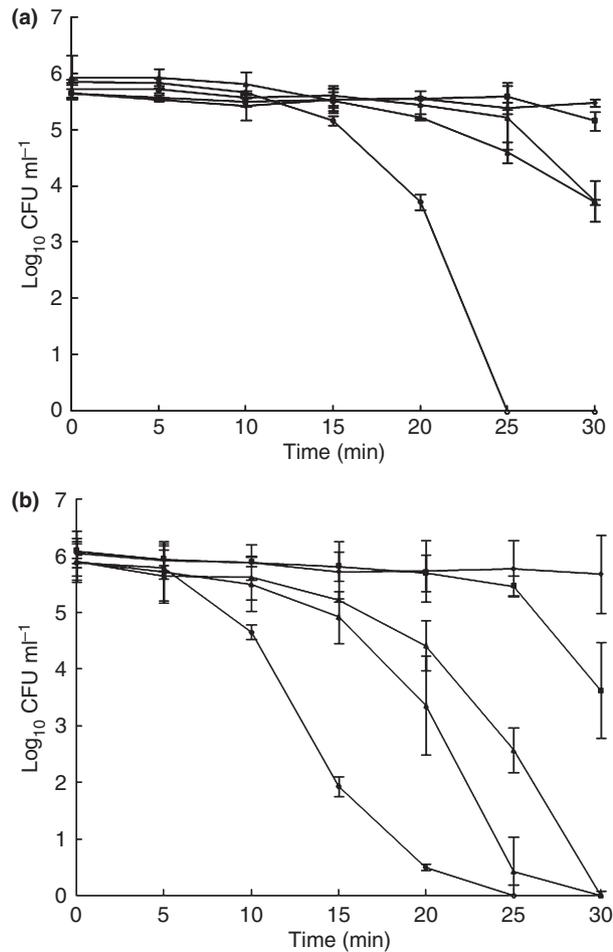


Figure 3 Effect of ozone concentration on the inactivation of *E. coli*. (a) At 0.06 l min^{-1} flow rate ozone concentrations: (\blacklozenge) $0.025 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$, (\blacksquare) $0.043 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$, (\blacktriangle) $0.056 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$, (\triangleleft) $0.072 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$, (\circ) $0.078 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$. (b) At 0.12 l min^{-1} flow rate ozone concentrations: (\blacklozenge) $0.03 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$, (\blacksquare) $0.048 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$, (\blacktriangle) $0.068 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$, (\triangleleft) $0.087 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$, (\circ) $0.098 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$. Error bars represent standard deviation.

Modelling of the inactivation kinetics and assessment of the process

Each individual experiment was fit to a Weibull model. The Weibull model adequately described the microbial inactivation (Fig. 5). The relationship between model parameters (α and β) and processing parameters of ozone flux ($f\text{O}_3$) and temperature (T) are shown in Fig. 6. The characteristic time relationship with the ozone flux through the reactor showed an increase in the inactivation rate as ozone flux increased until a flux of approximately 0.1 mg O_3 per processing minute per ml of reactor volume. The dependence of the characteristic time

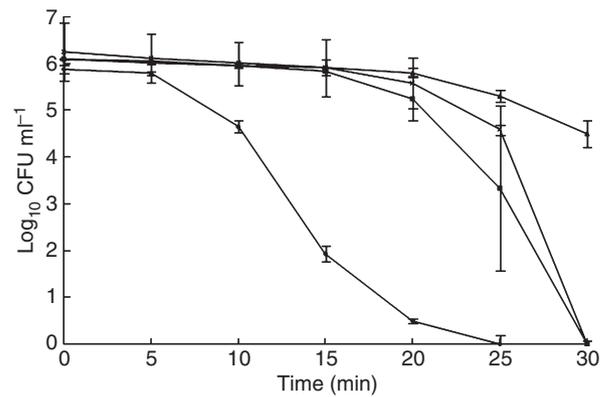


Figure 4 Effect of temperature on the efficacy of ozone inactivation of *E. coli*. (\blacklozenge) ambient, (\blacksquare) 20°C , (\blacktriangle) 25°C , (\times) 30°C . Error bars represent standard deviation.

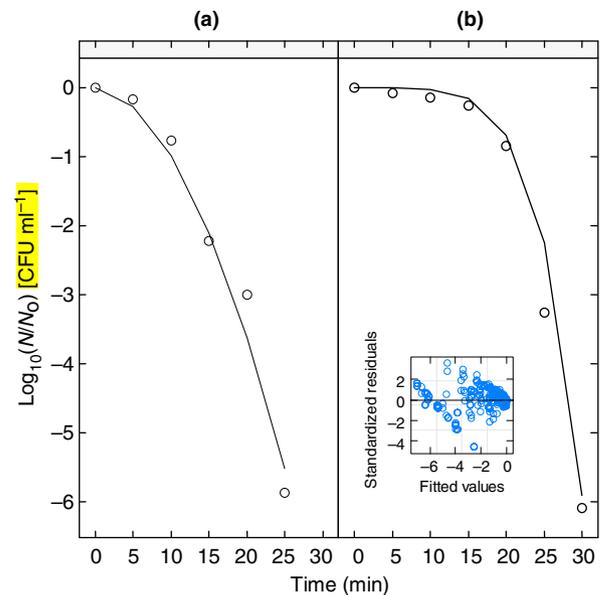


Figure 5 Typical Weibull model fit (continuous line) for the ozone inactivation experimental data. The insert of (b) shows the standardized residual vs the fitted values, indicating a satisfactory fit. (a) $0.145 \text{ mg ozone min}^{-1} \text{ ml}^{-1}$ at ambient temperature; (b) $0.087 \text{ mg ozone min}^{-1} \text{ ml}^{-1}$ at 20°C .

with temperature showed that lower processing temperatures achieved higher inactivation rates. The shape parameter β showed no clear dependence with $f\text{O}_3$, however, a sharp increase of β was found with increasing temperature. At higher processing temperatures the concavity of the Weibull curve will increase and the lag time prior to inactivation will be shorter. This effect is opposite to the effect that temperature has on $\log(\alpha)$, however, in the present study the temperature effect on $\log(\alpha)$

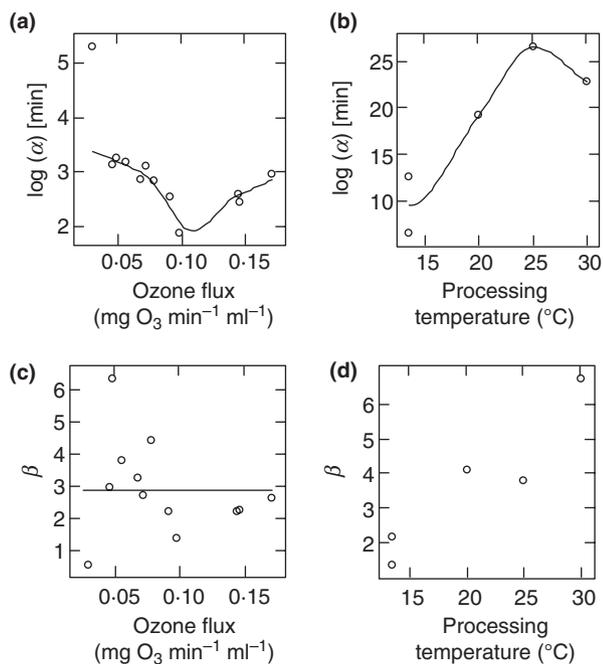


Figure 6 Relationship of the log of the characteristic time α (a) with the ozone processing conditions at ambient temperature and (b) with the processing temperature at $0.09 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$ and the shape parameter relationship β with (c) the ozone processing conditions at ambient temperature and (d) the temperature at $0.09 \text{ mg O}_3 \text{ min}^{-1} \text{ ml}^{-1}$ of reactor volume. The continuous line represents a smoothing of the estimates.

prevails and consequently at higher temperatures (up to 25°C) less inactivation was found.

Considering the evidence for curvature on the $\log(\alpha)$ and β values with ozone and temperature from Fig. 6, a model with quadratic effects of ozone and temperature on the characteristic time and shape parameter was built. Additional parameters of dependence of the $\log(\alpha)$ with the ozone concentration and the flow rate were found to be significant. Table 3 shows the estimated parameters from the Weibull model fit (All parameters were significant at $P < 0.05$).

Discussion

The extrinsic parameters investigated had significant influence on the efficacy of ozone. 5 log reductions were achieved in TSB, depending on the flow rate applied to the cell suspension. Others have reported on the effects of intrinsic factors on ozone inactivation including medium type and organic matter content. Ozone has a high oxidation potential, it reacts with micro-organisms as well as with other particles and compounds if placed in an environment such as food systems that are rich in organic matter (Kim *et al.* 1999). The survival curves of *E. coli* in

Table 3 Final candidate model for the inactivation of *E. coli*. All parameters significant at $P < 0.05$ level

Parameter	Estimates
$\log \alpha$	$1.7_{(0.3)} - 47_{(5)} \times f\text{O}_3 + 192_{(2.3)} \times f\text{O}_3^2 + 0.281_{(0.02)} \times T - 0.0053_{(0.0005)} \times T^2 + 1.1_{(0.2)} \times \text{flow rate} + 0.006_{(0.001)} \times [\text{Ozone}]$
β	$-5.2_{(1.1)} + 0.52_{(0.08)} \times T$
Model fit	
RMSE	0.732
SSQ	172.81
R^2	0.91
R^2_{adj}	0.90
N	115

RMSE, root mean square error; SSQ, sum of squares of data; R^2 , coefficient of determination; R^2_{adj} , adjusted R -square; n , number of experiments.

TSB treated with ozone showed a noticeable lag time before cell death was observed. Lag times were also observed in other studies, Chen *et al.* (1992) reported approximately 10 min lag time in 0.8% saline solution when *E. coli* was treated with $5 \text{ mg O}_3 \text{ l}^{-1}$ at a flow rate of 0.1 l min^{-1} . Williams *et al.* (2005), studied the inactivation of *E. coli* in orange juice, and found the efficacy of ozonation was reduced in the presence of ascorbic acid and organic matter. A defined lag time was also observed in the current work when treatment was carried out in TSB. The ozone flow rate was found to be an important factor for inactivation. This may be due to the effect of gas flow rates on bubble size. A possible explanation could be that the bubble size generated at the higher flow rates was too large. At high flow rates a small number of large bubbles are produced, which rise to the liquid surface quickly, thereby escaping the medium quickly. The resulting poor gas dissolution reduces the contact time, leading to a lower inactivation rate. At low flow rates, small bubbles are produced, however as the amount of ozone applied is low, the corresponding inactivation is slow.

Lag times tended to increase with increasing temperature. Herbold *et al.* (1989) showed that ozone inactivation of hepatitis A virus and *E. coli* was faster at 10°C than at 20°C . Ozone solubility in water is 13 times that of Oxygen at 0 – 30°C (Rice 1986). The solubility ratio for ozone increases as the temperature of water decreases (Bablon *et al.* 1991). Ozone decomposition is faster at higher water temperatures (Rice *et al.* 1981). As the temperature increases ozone becomes less soluble and less stable with an accompanying increase in the decomposition rate. Achen and Yousef (2001) treated *E. coli* contaminated apples with ozone at 4, 22 and 45°C , and observed that counts of the bacterium on the surface decreased by 3.3,

3.7 and 3.4 log₁₀-units, respectively. Steenstrup and Floros (2004), studied the effect of temperature (5–20°C) at 860 ppm (v/v) ozone and different gaseous ozone concentrations above 1000 ppm on inactivation of *E. coli* O157:H7 in apple cider and reported *D* values ranging from 0.6 to 1.5 min at 20°C and 5°C, respectively. The temperatures employed in this study ranged from ambient to 30°C. *Escherichia coli* is a mesophilic micro-organism and would not be inactivated at these temperatures in the absence of ozone. In the current study, a reduction >5 log cycles was achieved at ambient temperature (12–15°C) within 25 min. The antimicrobial activity of ozone decreased with increasing temperatures. This result showed that the efficacy of ozone inactivation depended on the medium type and organic matter content. Based on these investigations, to optimize the use of ozone for liquid systems, temperatures should be maintained at low to ambient, the maximum obtainable ozone concentration should be employed and the flow rate should be selected to maximize solubility thus minimizing gas escape from the free surface. Tiwari et al. (2008b,c, 2009) recently studied the effects of ozone on quality and nutritional parameters for a range of fruit juices, highlighting significant losses in nutritional quality which were dependent on ozone control parameters of ozone concentration and gas flow rate. However, achieving rapid microbial inactivation using optimized control parameters may mitigate losses in nutritional quality. Ozone inactivation efficacy will be dependent on the food system employed and various fruit juices should be investigated further. The ozone inactivation kinetics of *E. coli* was well described by the Weibull model with high values for R^2 , indicating goodness of fit. The inactivation of *E. coli* displayed a downward concavity, with a β parameter >1, indicating the susceptibility of remaining cells to the ozone treatment (van Boekel 2002).

The fitting of the microbial inactivation parameters with the process variables yielded a model that presented a dependence not only on the amount of ozone introduced per minute, but also a dependence on the total gas flux and the ozone concentration in the bubble. The final model points to a compromise region of intermediate ozone flow rates (around 0.1 mg O₃ min⁻¹ ml⁻¹) at ambient temperatures.

Conclusions

This work shows the optimized critical control parameters governing ozone inactivation of *E. coli* in a liquid system. Control of these parameters is essential for optimized inactivation to meet food safety requirements. Inactivation of *E. coli* was rapid at ambient temperature, this has the advantage of less energy consumption than traditional

thermal pasteurization processes, while minimizing the effects on quality parameters.

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