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Assessing the mechanism of microbial inactivation during ozone processing

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ABSTRACT

Ozone has numerous applications in food industry because of its advantages over traditional preservation techniques. Damage to cell membranes and cytoplasmic contents was proposed as involved in ozone inactivation but there is no available information concerning oxidative stress effect of ozone on regulated knockout genes and the protection or sensitivity of microbial mutants (lacking these genes) against ozone or ozone generated radicals. The aim of this work was to investigate the mechanism of action of ozone on microbial populations during the treatment of liquid food systems. *E. coli* BW25113 and its isogenic mutants in *soxR*, *soxS*, *oxyR*, *rpoS*, *dnaK* genes were treated with ozone at a previously optimized concentration of 6µg/mL for a period up to 4 min in a 100 mL bubble column. Ozone gas was generated using a corona discharge ozone generator. Oxygen was supplied via air cylinder and the flow rate (0.06 L/min) was controlled using a flow regulator. A significant effect of ozone exposure on microbial inactivation was observed while the cell membrane integrity and permeability were affected by ozonation. Scanning Electron Microscopy (SEM) analysis showed slightly altered cell surface structure. The results of this study suggest that cell lysis was not the primary mechanism of microbial inactivation. The absence of oxidative stress-related genes resulted in increased susceptibility of *E. coli* cells to ozone treatment suggesting that they play an important role for protection against the radicals produced by ozone. However, DnaK which has previously been shown to protect against oxidative stress did not protect against ozone treatment. Furthermore, RpoS was important for survival against ozone through an unidentified mechanism. This preliminary study provides important information about the mechanism through which ozonation acts against microorganisms for the production of safe liquid food products.

Keywords: Ozone; non thermal technology; E. coli; microbial inactivation kinetics

INTRODUCTION

Ozone is a powerful antimicrobial agent due to its potential oxidizing capacity. Ozone decomposes rapidly to molecular oxygen leaving no residues, thereby, making it an environmentally friendly and safe antimicrobial agent for use in the food industry [1]. The bacterial cell surface has been suggested as the primary target of ozonation [2]. The decomposition of ozone results in the generation of superoxide, hydroperoxyl, hydroxyl radicals [3-4]. However, microorganisms develop mechanisms to counteract the lethal effects of the reactive oxygen species [5]. Up to now there is really no extensive information on the main cellular target of ozone treatment although damage to cell membranes and cytoplasmic contents has previously been proposed [6-8]. In this study, with the use of deletion *E. coli* mutants in *soxR*, *soxS*, *oxyR*, *rpoS* and *dnaK* which have been shown to play an important role in the protection against reactive oxygen radicals, the nature of the ozone treatment and its cellular targets was investigated.

MATERIALS & METHODS

The bacterial strains used in this study were Δ*soxR* (*E. coli* JW 4024), Δ*soxS* (*E. coli* JW 4023), Δ*oxyR* (*E. coli* JW3933), Δ*rpoS* (*E. coli* JW 5437), Δ*dnaK* (*E. coli* JW0013) mutants and their isogenic parent *E. coli* BW 25113 [9]. All strains were obtained from the National BioResource Project, Japan (NIG, Japan). Strains were maintained as frozen stocks at -70ºC in the form of protective beads, which were plated onto tryptic soy agar (TSA) and incubated overnight at 37ºC to obtain single colonies before storage at 4ºC. Working cultures were prepared by inoculating a single colony into tryptic soya broth without glucose (TSB-G) followed by overnight incubation at 37ºC. Cells grown in TSB-G were harvested by centrifugation and cell pellet was washed twice with sterile saline. The bacterial density was determined by measuring absorbance at 550 nm

using McFarland standard (BioMérieux, Marcy -l'Etoile, France). The inoculum was then suspended in saline to obtain approximately 10^8 CFU/mL.

Ozone gas was generated using a corona discharge ozone generator (Model OL80, Ozone services, Burton, Canada) and oxygen was supplied using air cylinder. *E. coli* strains that were suspended in saline were subjected to ozone treatment by passing ozone gas through the cell suspension in an ozone bubble column. A flow rate of 0.06 L/min with an ozone concentration of 6 µg/mL recorded using an ozone analyzer (built in ozone module OL80A/DLS, Ozone Services) was applied for each treatment. Samples were removed at 30 sec intervals and kept on ice until further analysis. The plating was carried out after the end of the treatment. In order to obtain a low microbial detection limit, 1 mL was spread onto TSA plates as described in EN ISO 11290-2 method [10]. The limit of detection was 1 log CFU/mL. Plates were incubated at 37ºC for 24 h and colony forming units were counted. Results were reported as Log_{10} CFU/mL. The GInaFiT tool was employed to perform the regression analysis of the microbial inactivation data [11]. The biphasic eqn 1 [12] was selected based on preliminary statistical comparison of the different inactivation models described in GInaFiT software [11]:

$$\log_{10}(N) = \log_{10}(N_0) + \log_{10}\left(f \times e^{(-k_{\max 1} \times t)} + (1-f) \times e^{(-k_{\max 2} \times t)}\right) \quad [1]$$

where, N is the number of microorganisms, N_0 CFU/mL is the initial number of microorganisms, f is the fraction of initial population in a major subpopulation, 1-f is the fraction of initial population in a minor subpopulation, k_{max1} and k_{max2} are the parameters that determine the inactivation rate.

The time required to achieve a 5 log reduction (t_{5d}) was calculated using the Solver in Microsoft Excel (Microsoft Corporation, USA). For statistical analysis, means were compared using ANOVA followed by LSD testing at $p < 0.05$ level (SPSS, version 15.0). Membrane integrity was examined by determination of the release of material absorbing at 260 nm and 280 nm [13]. Ozonated samples extracted at preset time intervals were centrifuged at 15,000 RPM for 20 min at 4ºC and the absorbance values at 260 nm and 280 nm were recorded using a UV spectrophotometer. Cell membrane permeability was determined using a hydrophobic probe, 1-N-Phenylnaphthylamine (NPN). Ozone treated *E. coli* cultures (160 µL) were pipetted into microtitre plate wells to which 40 µL of 100 µM NPN was added, yielding an end concentration of 20 µM NPN. Immediately after mixing, plates were read on a Bio Tek Synergy HT fluorescence plate reader (excitation wavelength, 360/40 nm and an emission wavelength, 460/40 nm). Samples for scanning electron microscopy (SEM) were prepared according to the procedure employed by Thanomsub et al. [14] with minor modification.

RESULTS & DISCUSSION

There was a significant effect on microbial inactivation due to the ozone exposure. The parent strain and the Δ*dnaK* mutant were comparatively less susceptible to ozone treatment than Δ*soxR*, Δ*soxS*, Δ*oxyR* and Δ*rpoS* mutants as illustrated from the t_{5d} values ($p<0.05$) (Table 1) (see Fig. 1 for representative kinetic studies). The lowest t_{5d} values for Δ*soxR*, Δ*soxS*, Δ*oxyR* and Δ*rpoS* mutants highlight the importance of oxidative stress related genes for the protection of *E. coli* against ozone treatment. The SoxRS regulon (superoxide response regulon) has previously been shown to play an important role in the protection against ozone treatment in *E. coli* [15]. The genes of SoxRS regulon are directly responsible for the removal of superoxide anions or repair of superoxide damaged macromolecules such as DNA. The sensitivity of Δ*soxR* and Δ*soxS* mutant indicates that one of the main cellular targets of ozone treatment is the DNA. OxyR is another transcriptional regulator required for the induction of hydrogen peroxide (H_2O_2) inducible genes like *katG*, *ahpCF grxA* [16]. Strains with *oxyR* deletions are unable to induce this regulon and are hypersensitive to H_2O_2 [17]. Hence, the absence of the H_2O_2 inducible gene activator in Δ*oxyR* mutant resulted in increased sensitivity to ozone. One of the most important regulators of stress genes involved in general stress resistance is RpoS. However, until now it has not been shown if it plays any role in protecting the cells against ozone treatment. Here we demonstrate that RpoS has an important role in the resistance of *E. coli* against ozone. This finding can lead to further research focusing on the identification of the specific genes of the RpoS regulon which are important for protection against ozone treatment.

When *E. coli* strains were treated with ozone, the absorbance at 260 nm increased immediately after 30 sec of treatment irrespective of the parent strain or mutant strains studied. The absorbance at 280 nm after ozone treatment was not significant for all the strains studied. In all cases the 260/280 ratio remained constantly at

levels equal or above 2. For all *E. coli* strains, ozone treatment of 30 sec resulted in increased NPN uptake (Table 1) and further exposure to ozone did not give a significant increase in fluorescence. For SEM analysis, time 0 and 30 sec samples were chosen and the analysis was performed for the parent strain and two of the most sensitive mutant strains; Δ*oxyR* and Δ*soxR*. Detailed observation of *E. coli* cells after SEM analysis showed slightly altered cell surface structure but to a less extent compared to the untreated cells (Fig. 2 for representative images). The surface of ozone treated *E. coli* appeared slightly rough compared to the non-ozonated cells. Release of intracellular components after ozonation was not observed for all *E. coli* strains in the current study. This is obvious from the fact that the absorbance observed at 260 nm was significantly lower than that reported previously [18-19]. Furthermore, the constant 260/280 ratio at levels equal or above 2 suggests that no proteins were released during ozone treatment. The above is also confirmed by the SEM results which do not show any significant damage of the cells that can allow high protein leaking. Similar observation could be made from the results obtained with the use of NPN for assessing the cell membrane permeability. The current results also suggest that proteins do not seem to be the main cellular target of ozone treatment. Additional studies will be focusing on identifying the role of DnaK, if any, against specific radicals.

Table 1: Inactivation parameters obtained following regression analysis with a biphasic model, t_{5d} values and relative fluorescence values for *E. coli* strains (Different letters indicate a significant difference at the 0.05 level between each strain).

***E. coli* Strain**	k_{max1}	k_{max2}	**RMSE**	t_{5d} (sec)	**Relative NPN fluorescence**
BW 25113 (parent)	0.340±0.150	0.021±0.001	0.11	204.96	44±8.71
Δ*rpoS*	0.275±0.03	0.026±0.004	0.23	129.19	40±3.60
Δ*soxR*	0.331±0.06	0.032±0.004	0.23	92.06	53.33±12.4
Δ*soxS*	0.287±0.03	0.025±0.004	0.22	117.08	45±7.5
Δ*oxyR*	0.317±0.04	0.031±0.003	0.19	107.06	40±5.40
Δ*dnaK*	0.248±0.03	0.017±0.002	0.15	258.61	38.9±9.8

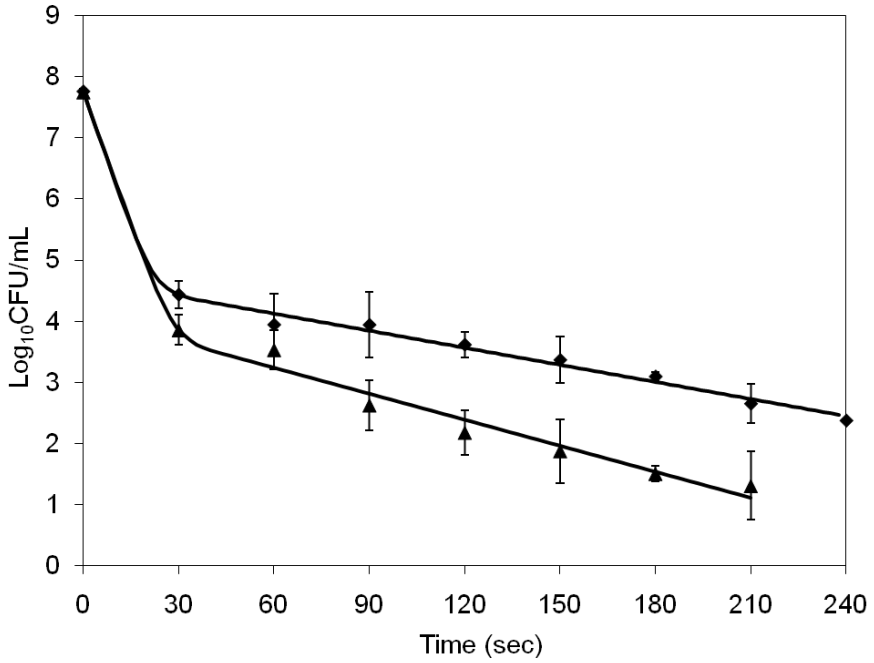

Figure 1: Effect of ozone on microbial inactivation kinetics of *E. coli* strains

♦ *E. coli* BW 25113 ▲ Δ*soxR*

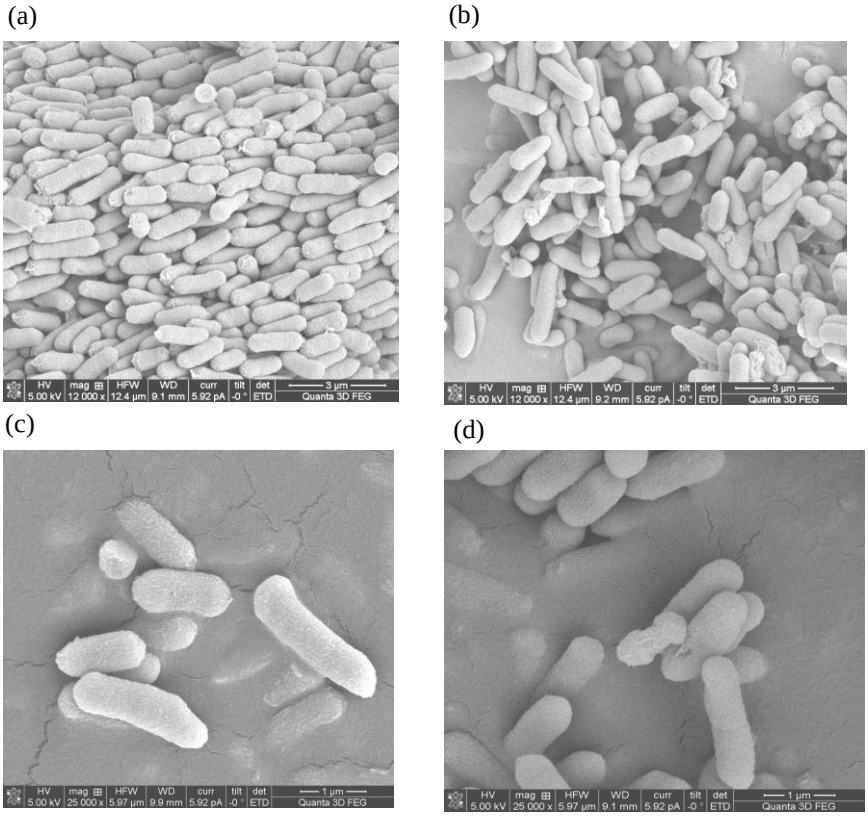

Figure 2: Scanning electron micrograph of untreated and ozone treated *E. coli.*
(a) *E. coli* BW 25113- untreated
(b) *E. coli* BW 25113- ozone treated (30 sec)
(c) Δ*soxR*- untreated
(d) Δ*soxR*- ozone treated (30 sec)

In the current study, and based on all the applied analysis, it was observed that cell lysis was not the main mechanism of inactivation. The ability of ozone to diffuse through the membrane appears to damage the cell constituents, thereby negatively impacting on their metabolic activity and consequently leading to the final inactivation of the cells.

CONCLUSION

In the present work a primary step was made to elucidate the nature of the ozone treatment and the cellular targets generally involved in the inactivation of cells by ozone. From the present results it was evident that cell lysis was not the major mechanism of inactivation. Experiments performed with mutants in genes conferring protection against oxidative stress demonstrated the important role of the SoxRS and the OxyR regulon in protection against ozone treatment. The role of specific cellular targets, as well as the identification of genes from the RpoS regulon playing a role in protection against ozone treatment is of further investigation.

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