2011

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Recommended Citation

Bourke, P. et al. (2011) Assessing the Microbial Oxidative Stress of Ozone: Significant Role of the Oxidative Stress Proteins in the Survival of E. coli in Ozone Treatment. *Journal of Applied Microbiology,* vol.111 (1), 2011, p.136- 44.doi:10.1111/j.1365-2672.2011.05021.x DOI:10.1111/j.1365-2672.2011.05021.x

Funder: National Development Plan, Dept of Agriculture, Food & Fisheries

Assessing the microbial oxidative stress of ozone: significant role of the oxidative stress proteins in the survival of *E. coli* in ozone treatment

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Abstract

Aims: To investigate the effect of the oxidative stress of ozone on microbial inactivation, cell membrane integrity, membrane permeability and morphology changes of *Escherichia coli* during ozone treatment.

Methods and Results: *E. coli* BW 25113 and its isogenic mutants in *soxR, soxS, oxyR, rpoS, dnaK* genes were treated with ozone at a concentration of 6 µg mL^{-1} for a period up to 4 min. A significant effect of ozone exposure on microbial inactivation was observed. After ozonation, minor effects on the cell membrane integrity and permeability were observed. Scanning Electron Microscopy (SEM) analysis showed slightly altered cell surface structure.

Conclusions: The results of this study suggest that cell lysis was not the major mechanism of microbial inactivation. The deletion of oxidative stress-related genes resulted in increased susceptibility of *E. coli* cells to ozone treatment, implying 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 in this study. Furthermore, RpoS was important for survival against ozone through as of yet unidentified mechanism.

Significance and impact of the study: This study provides important information about the role of oxidative stress related proteins in *E. coli* survival during ozonation.

Keywords: *Escherichia coli,* ozone, SoxRS, OxyR, DnaK, RpoS, microbial inactivation

Introduction

Ozone is a powerful antimicrobial agent due to its potential oxidizing capacity. Previous studies have reported that ozone effectively inactivates bacteria in water (Levadnaya *et al*. 2009), vegetables, fruits (Selma *et al*. 2008), apple juice (Patil *et al*. 2010). 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 (Kim *et al*. 1999). It is also an unstable compound with a relatively short life; shorter half-life in water (20 min) than in air (3 days) at 20ºC. Different factors such as temperature, pH, and organic matter influence ozone decomposition. The progressive oxidation of vital cellular components by ozone is attributed to its antimicrobial properties. The bacterial cell surface has been suggested as the primary target of ozonation. Ozone oxidizes sulfhydryl groups, amino acids of enzymes, peptides and proteins to shorter peptides while it also oxidizes polyunsaturated fatty acids to acid peroxides (Victorin 1992).

The decomposition of ozone results in the generation of superoxide radicals ($^{\bullet}O_2^-$), hydroperoxyl radicals ($HO_2^{\bullet}$), hydroxyl radicals ($^{\bullet}OH$) (Adler and Hill 1950; Hoigné and Bader 1975). However, microorganisms have developed mechanisms such as the superoxide dismutases, reductases, peroxidises and catalases to counteract the lethal effects of the reactive oxygen species (Imlay 2008).

In *E. coli*, two such mechanisms are SoxR and OxyR which are redox responsive transcription regulators that have been well described (Pomposiello and Demple 2001). Both regulators are induced in the presence of $^{\bullet}O_2^-$ radicals (Greenberg *et al*. 1990) and activate various genes like *soxS* and *sod* (Pomposiello and Demple 2001) which in turn confer protection against these radicals. Several of these genes confer protection through DNA repair or removal of the radicals (Pomposiello and Demple

2001). The DnaK and RpoS are two regulators of general stress genes which although are not dedicated mechanisms of protection against oxidative radicals have been previously shown to confer protection against them (Delaney 1990; Rockabrand 1995; Loewen *et al*.1998). Similar radicals might be produced during ozone treatments and therefore these genes are expected to play an important role in protection of cells against this technology.

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 (Scott and Lesher 1963; Mudd *et al*. 1969; Pryor *et al*. 1991). In this study, with the use of deletion mutants in *soxR*, *soxS*, *oxyR*, *rpoS* and *dnaK* which have been shown to play an important role in the protection against reactive oxygen radicals, we attempted to investigate for first time the nature of the ozone treatment and its cellular targets. This work will enable us to further enhance understanding of the mechanism of action of ozone treatments to bactericidal effects.

Materials and methods

Bacterial strains and cultural conditions

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 (Baba *et al*. 2006). 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, Scharlau Chemie, Barcelona, Spain) 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) (TSB without dextrose, Scharlau Chemie) followed by overnight incubation at 37ºC.

Preparation of cell suspensions

Cells grown in TSB-G were harvested by centrifugation at 10,000 rpm (equivalent to 8720 x g) for 10 min at 4ºC. The cell pellet was washed twice with sterile saline, 0.85% (Sodium chloride, Scharlau Chemie). It was then re-suspended in saline and 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^{-1}.

Ozone treatment

Ozone gas was generated using a corona discharge ozone generator (Model OL80, Ozone services, Burton, Canada). Oxygen was supplied via air cylinder (Air Products Ltd., Dublin, Ireland) and the flow rate was controlled using a flow regulator. A flow rate of 0.06 L min^{-1} with an ozone concentration of 6 µg mL^{-1} was applied for each treatment. Conditions chosen were appropriate for the collection of complete kinetic responses in order to characterise accurately the microbial resistance of the studied strains. Ozone treatments at higher concentration (>6 µg mL^{-1}) resulted in rapid inactivation of bacterial cells and inactivation kinetics could not be performed. Ozone concentration was recorded using an ozone analyzer (built in ozone module OL80A/DLS, Ozone services, Burton, Canada) during ozone treatment in saline suspension. Excess ozone was destroyed by an ozone destroyer unit (OzoneLab™ Catalytic ozone destructor), which neutralizes ozone into oxygen using an organic catalyst prior to release. Ozone treatment of *E. coli* suspended in saline was carried out for 4 min with sampling intervals of 30 sec. Samples removed for analysis were immediately kept on ice and the plating was carried out after the end of the treatment

(after 240 sec). All experiments were performed in triplicate. Three independent ozone inactivation experiments for every strain using individual freshly prepared inoculum (10^8 CFU mL^{-1}) for each experiment were performed.

Determination of microbial inactivation

The effect of the ozone treatment on the microbial inactivation of each chosen strain was determined in terms of reduction of viable (culturable) counts over time. Samples (1 mL) were withdrawn, serially diluted in maximum recovery diluent (MRD, Scharlau Chemie, Spain) and 0.1 mL aliquots of appropriate dilutions were surface plated onto TSA. In order to obtain low microbial detection limits, 0.1 mL or 1 mL of the treated sample was spread onto TSA plates as described in EN ISO 11290-2 method (ISO 11290-2 1998). The limit of detection was 1 log CFU mL^{-1}. Plates were incubated at 37ºC for 24 h and colony forming units were counted. Results were reported as Log_{10} CFU mL^{-1}. The possibility of recovery of injured cells was taken into account by further incubating the plates for 2-3 days to detect possible increase in formation of visible colonies.

Inactivation kinetics

The inactivation kinetics of *E. coli* strains in saline showed a characteristic non-linear behaviour (refer to Results). This behaviour was described by the biphasic model and is given by eqn 1 (Cerf 1977). The GInaFiT tool was employed to perform the regression analysis of the microbial inactivation data (Geeraerd *et al*. 2005). The biphasic eqn (1) was selected based on preliminary statistical comparison of the different inactivation models available at GInaFiT:

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

where, k_{max1} and k_{max2} are the parameters that determine the inactivation rate.

The numerical values of f, k_{max1} and k_{max2} were used to calculate the time required to achieve a reduction by 5 log cycles (t_{5d}) using the Solver in Microsoft Excel (Microsoft Corporation, USA) by equalizing $\log_{10}(N_0)-\log_{10}(N)=5$.

In 2001, the U.S. Food and Drug Administration (FDA), published a final rule requiring fruit juice producers to achieve a 5-log reduction in critical pathogen levels (USFDA 2001). Therefore, a reduction of 5 log was considered in the present study. For statistical analysis, the t_{5d} values were calculated for each strain and average values and standard deviations of the replicated studies were determined. Means were compared using ANOVA followed by LSD testing at $p < 0.05$ level (SPSS, version 15.0).

Determination of cell membrane integrity

Membrane integrity was examined by determination of the release of material absorbing at 260 nm and 280 nm (Virto *et al*. 2005). Ozonated samples extracted at preset time intervals were centrifuged at 15,000 rpm for 20 min at 4ºC. Supernatant (200 µL) was added to a 96-well plate (UV–transparent flat-bottom microplates, Corning-Costar Cat. No. 3635; Fisher Scientific, Ireland), and absorbance values at 260 nm and 280 nm were recorded using a UV spectrophotometer (Synergy HT; Bio-Tek, USA). Controls included (i) *E. coli* in saline and (ii) saline only. Three independent experiments were performed and triplicate samples were analyzed. The absorbance values (for cell free supernatant of untreated and ozone treated samples) were subtracted with the simultaneously recorded value of cell free supernatant of saline sample.

Determination of cell membrane permeability

Cell membrane permeability was determined using a hydrophobic probe, 1-N-Phenylnaphthylamine (NPN). The quantum yield of NPN is greatly increased in a

glycerophospholipid environment compared with an aqueous environment (Träuble and Overath 1973; Liu *et al*. 2004). A 100 mM stock solution of NPN in acetone was diluted to a concentration of 100 µM in saline. Ozone treated *E. coli* cultures (160 µL) were pipetted into microtitre plate wells (black; Nunc) to which 40 µL of 100 µM NPN (Sigma-Aldrich, Dublin, Ireland) 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). Controls included (i) *E. coli* in saline with NPN and (ii) saline with NPN. Three independent experiments were performed and triplicate samples were analyzed. The fluorescence values were subtracted with the simultaneously recorded value of the untreated cell sample (control sample) in the presence of 20 µM NPN.

Scanning Electron Microscope (SEM) imaging

Samples for SEM were prepared according to the procedure employed by Thanomsub *et al*. (2002) with minor modification. In detail, control (untreated) and treated samples of the parent and Δ*soxR* and Δ*oxyR* sensitive mutant strains were collected at intervals of 0 and 30 sec, as other assays indicated rapid population reduction and release of intracellular components within a period of 30 sec. Samples were concentrated by centrifugation at 10,000 rpm for 10 min. The supernatant was discarded and the cells were fixed in ice-cold 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH=7·4) for 2 h. Cells were then washed with the same buffer three times and were then fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate for 2 h at 4°C. Cells were then washed once with the same buffer followed by three washes with distilled water. Samples were dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95% to 99.5%). The dehydrated samples were freeze dried

(Labconco, FreeZone 6, Mason Technology, Dublin, Ireland), mounted on stubs using double sided carbon tape, and sputter coated with Au, using a Emitech K575X Sputter Coating Unit, to prevent surface charging by the electron beam. Samples were then sputter coated at a vacuum of 5 x 10^{-3} mbar around 30 sec resulting in a coating of 10 nm. Further on the samples were examined using a FEI Quanta 3D FEG Dual Beam SEM (FEI Ltd, Hillsboro, USA) at 5KV. SEM analysis was employed to image the damage or alteration in cell surface structure of bacterial cell population, after ozone treatment.

Results

Effect on microbial inactivation kinetics

There was a significant effect on microbial inactivation due to the ozone exposure and for all strains the microbial kinetics exhibited biphasic behaviour (Fig. 1). The inactivation parameters were estimated by eqn (1) and the t_{5d} values (Table 1) were calculated as described in the Materials and Methods. The parent strain and the Δ*dnaK* mutant were comparatively less susceptible to ozone treatment than Δ*soxR*, ΔsoxS, Δ*oxyR* and Δ*rpoS* mutants as illustrated from the t_{5d} values ($p<0.05$) (Table 1). No significant difference between the four sensitive mutant strains was recorded. However, for most of the strains, a 5 log cycle reduction was achieved within 4 min. The incubation of the plates for additional 2-3 days at 37ºC did not result in increase of the number of colony forming units.

Effect on cell membrane integrity and membrane permeability

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 (Fig. 2a). The maximum absorbance at 260 nm was noticeable for Δ*oxyR* and Δ*rpoS* mutants. Δ*oxyR* mutant showed significantly higher absorbance values at 260

nm (p<0.05) after 30 and 60 sec of ozone treatment compared to parent and other mutant strains studied. Ozone treatment of 90 sec showed significantly higher absorbance values (p<0.05) at 260 nm for Δ*oxyR* and Δ*rpoS* mutants. Ozonation for the longest treatment time (240 sec) showed significantly lower absorbance values for parent, Δ*soxR*, Δ*soxS* and Δ*dnaK* mutants compared to Δ*oxyR* and Δ*rpoS* mutants. The absorbance at 280 nm after ozone treatment was not significantly noticeable for all the strains studied. The 260/280 ratio remains constantly at levels equal or above 2 for all studied strains.

The uptake of NPN by *E. coli* strains after 30 sec of ozone treatment is shown in Table 1. For all *E. coli* strains, ozone treatment of 30 sec resulted in increased NPN uptake. Further exposure to ozone did not result in a significant increase in fluorescence**.**

SEM examination of ozone treated *E. coli*

All *E. coli* strains showed a rapid reduction in population and a release of intracellular components following 30 sec of ozone treatment. Therefore, 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 and damage to the cell surface but to a less extent compared to the untreated cells (Fig. 3). The surface of ozone treated *E. coli* appeared slightly rough compared to the non-ozonated cells.

Discussion

In this work for the first time we attempted to get information regarding the nature and the main cellular targets of ozone treatment by the use of carefully selected mutant strains. Interestingly, the same intensity of ozone treatment had different

effects on mutants in genes which have previously shown to play an important role in oxidative stress. These could lead us to some interesting conclusions regarding ozone treatment.

The lowest t_{5d} for Δ*soxR*, Δ*soxS*, Δ*oxyR* and Δ*rpoS* mutants highlights 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* (Jimenez-Arribas *et al*. 2001). The SoxR is the activator which in its oxidised form enhances transcription of *soxS* that encodes for a transcriptional activator of several genes of the SoxRS regulon (e.g. *sodA* and *nfo*; Pomposiello and Demple 2001). These genes are directly responsible for the removal of superoxide anions or repair of superoxide damaged macromolecules but mainly DNA. These could explain why both Δ*soxR* and Δ*soxS* mutant were sensitive to ozone treatment. In addition, it provides strong evidence that one of the main cellular targets of ozone treatment is the DNA. Previous studies also reported ozone causes damage to DNA which if unrepaired results on extensive breakdown of DNA in *E. coli* and consequently loss of cell viability (Hamelin and Chung 1974; Hamelin *et al*. 1977; 1978). The damage of the chromosomal DNA might be one of the reasons for inactivation of *E. coli* by ozone (Ishizaki *et al*. 1987).

OxyR is another transcriptional regulator required for the induction of hydrogen peroxide (H_2O_2) inducible genes like *katG, ahpCF grxA* (Zheng *et al*. 1998, Zheng *et al*. 2001). The interaction of H_2O_2 with iron localized along the phosphodiester backbone of nucleic acids leads to cell death upon exposure to H_2O_2 (Storz and Imlay 1999). Strains with *oxyR* deletions are unable to induce this regulon and are hypersensitive to H_2O_2 (Christman *et al*. 1989). Hence, the absence of the H_2O_2

inducible gene activator in Δ*oxyR* mutant resulted in increased sensitivity to ozone (refer to Table 1 and Fig.1).

One of the most important regulators of stress genes involved in general stress resistance is RpoS. The RpoS subunit of RNA polymerase is the master regulator of general stress response in *E. coli*, positively regulating more than 500 (10%) genes (Hengge 2009). RpoS is known to regulate the expression of genes which are important against oxidative stress (*katP, ahpCF*) (Loewen *et al*. 1998). However, until now it has not been shown if it plays any role in protecting the cells against ozone treatment. Here we demonstrate for first time that RpoS plays an important role in the resistance of *E. coli* against ozone. This result opens the door for further research which could focus on the identification of the specific genes of the RpoS regulon which are important for protection against ozone treatment.

The parent strain as well as the Δ*dnaK* mutant was less susceptible to ozone treatment than the other mutants. This observation is highly interesting since DnaK has previously been shown to play an important role in the resistance of *E. coli* cells against H_2O_2 (Delaney 1990; Rockabrand 1995) mainly through the protection of proteins from oxidative damage (Echave 2002). The current results suggest that proteins do not seem to be the main cellular target of ozone treatment. Another explanation could be that the generated radical species and the intensity of the treatment led to the rapid inactivation of DnaK in the wild type. This has been previously observed during severe oxidative stress (but at experiments performed) at high temperatures (Winter *et al*. 2005). In that study it was suggested that severe oxidative stress rather rendered DnaK thermolabile at high temperatures than causing its inactivation *per se*. However, no increase in the temperature was observed in these experiments suggesting that the above did not take place in our experiments.

Additional studies are required to further identify the nature of ozone treatment, its cellular targets and the role of DnaK, if any, against specific radicals.

Macromolecules such as proteins and lipids have different susceptibilities to oxidative damage within a range of time scales which makes the characterisation of the oxidative stress mechanism a complicated task (Semchyshyn *et al*. 2005). 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 (Komanapalli and Lau 1996; Curtiellas *et al*. 2005). Furthermore, the 260/280 ratio remains constantly at levels equal or above 2 which suggests that no proteins were released during ozone treatment. However, despite the above possible damage induced by ozone does not seem to be so significant that would allow proteins to leak out of the cell. The above statement is also confirmed by the SEM results which do not show any significant damage of the cells while the same could be concluded by the results obtained with the use of NPN. As White, (1999) reported in the presence of ozone, the macromolecules released from the cell are further cleaved and oxidized, resulting in much smaller molecules. Cho *et al*. (2010) also concluded that proteins degrade due to the reaction for a number of disinfectants in the order of ozone, chlorine dioxide, free chlorine and UV and that the amount of proteins released from *E. coli* inactivation is also dependent on the degradation reaction.

The parent and mutant *E. coli* strains showed increased uptake of NPN followed by ozonation which is normally excluded by Gram negative bacteria. Disruption of the outer membrane weakens the bacteria and allows the permeability to large, hydrophobic molecules (Murray *et al*.2009) like NPN indicating damage to the outer membrane (Helander and Mattila-Sandholm 2000). It is evident from the current

results that the membrane damage by ozonation is less when compared with that reported by using chitosan (Liu *et al*. 2004) and lactic acid (Alakomi *et al*. 2000) as antimicrobial agents.

Two main theories appear in the literature about the microbial inactivation mechanism during ozonation: (i) cell lysis (Scott and Lesher 1963; Mudd *et al*. 1969; Hamelin *et al*. 1978; Hunt and Marinas 1999; Thanomsub *et al*. 2002), (ii) interaction with cell constituents (Ingram and Haines 1949; Hamelin *et al*. 1977, 1978; Ishizaki *et al*. 1987). According to the latter, ozone diffuses through the membrane and react with cell (vital) component. Similarly, Perrich *et al*. (1975) concluded that cell lysis was not the main mechanism for *E. coli* inactivation and that the cells remained morphologically intact after inactivation.

In the current study, and based on the SEM 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.

Conclusions

From the present results it was evident that cell lysis was not the major mechanism of inactivation shown in this study. 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. DnaK which was shown previously to play a role in H_2O_2 resistance did not appear to protect against ozone. 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. 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.

Acknowledgement

Funding for this research was provided under the National Development Plan 2000-2006, through the Food Institutional Research Measure, administered by the Department of Agriculture, Fisheries & Food, Ireland.

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Table 1: The t_{5d} (time to achieve 5-log reduction) values and relative fluorescence values for *E. coli* strains (Different letters indicate a significant difference at the 0.05 level between each strain) obtained after ozone treatment at 6 µg mL^{-1} in saline solution.

E. coli Strain	k_{max1}	k_{max2}	RMSE	t_{5d} (sec)	*Relative NPN fluorescence (after 30 sec of ozone treatment)
BW 25113 (Parent strain)	0·3403±0·150	0·0214±0·001	0·11	204·96[a]	44±8·71
Δ*rpoS*	0·2915±0·080	0·0332±0·004	0·33	128·08[b]	40±3·60
Δ*soxR*	0·3436±0·079	0·0345±0·003	0·22	98·64[b]	53·33±12·4
Δ*soxS*	0·2928±0·040	0·0283±0·003	0·23	120·06[b]	45 ±7·5
Δ*oxyR*	0·3168±0·041	0·0314±0·002	0·16	108·18[b]	40±5·40
Δ*dnaK*	0·2478±0·027	0·0175±0·002	0·15	258·61[a]	38·9±9·8

*The NPN uptake values represent fluorescence units (±SD) after subtraction of cell control before ozone treatment.

RMSE: Root mean square error

k_{max1} and k_{max2}: parameters determining inactivation rate

t_{5d}: time required to achieve 5-log reduction

Figure Legends

Figure 1 Effect of ozone at a concentration of 6 µg mL^{-1} on microbial inactivation kinetics of different *E. coli* strains in saline solution

(a) *E. coli* BW 25113 (parent strain)

(b) Δ*rpos*

(c) Δ*soxR*

(d) Δ*soxS*

(e) Δ*oxyR*

(f) Δ*dnaK*

Figure 2 Release of cellular material absorbing at a) 260 nm and b) 280 nm from *E. coli* cells treated with ozone at a concentration of 6 µg mL^{-1} in saline solution: ♦- parent strain BW 25113; ■- Δ*rpos*; ▲-Δ*soxR*; Δ-Δ*soxS*; ○-Δ*oxyR*; ●-Δ*dnaK*

Figure 3 Scanning electron micrograph of untreated and ozone treated (at a concentration of 6 µg mL^{-1} in saline solution) *E. coli.* Damaged or altered cells after ozone treatment are indicated by arrows

(a) *E. coli* BW 25113- untreated

(b) *E. coli* BW 25113- ozone treated (30 sec)

(c) Δ*oxyR*- untreated

(d) Δ*oxyR*- ozone treated (30sec)

(e) Δ*soxR*- untreated

(f) Δ*soxR*- ozone treated (30 sec)

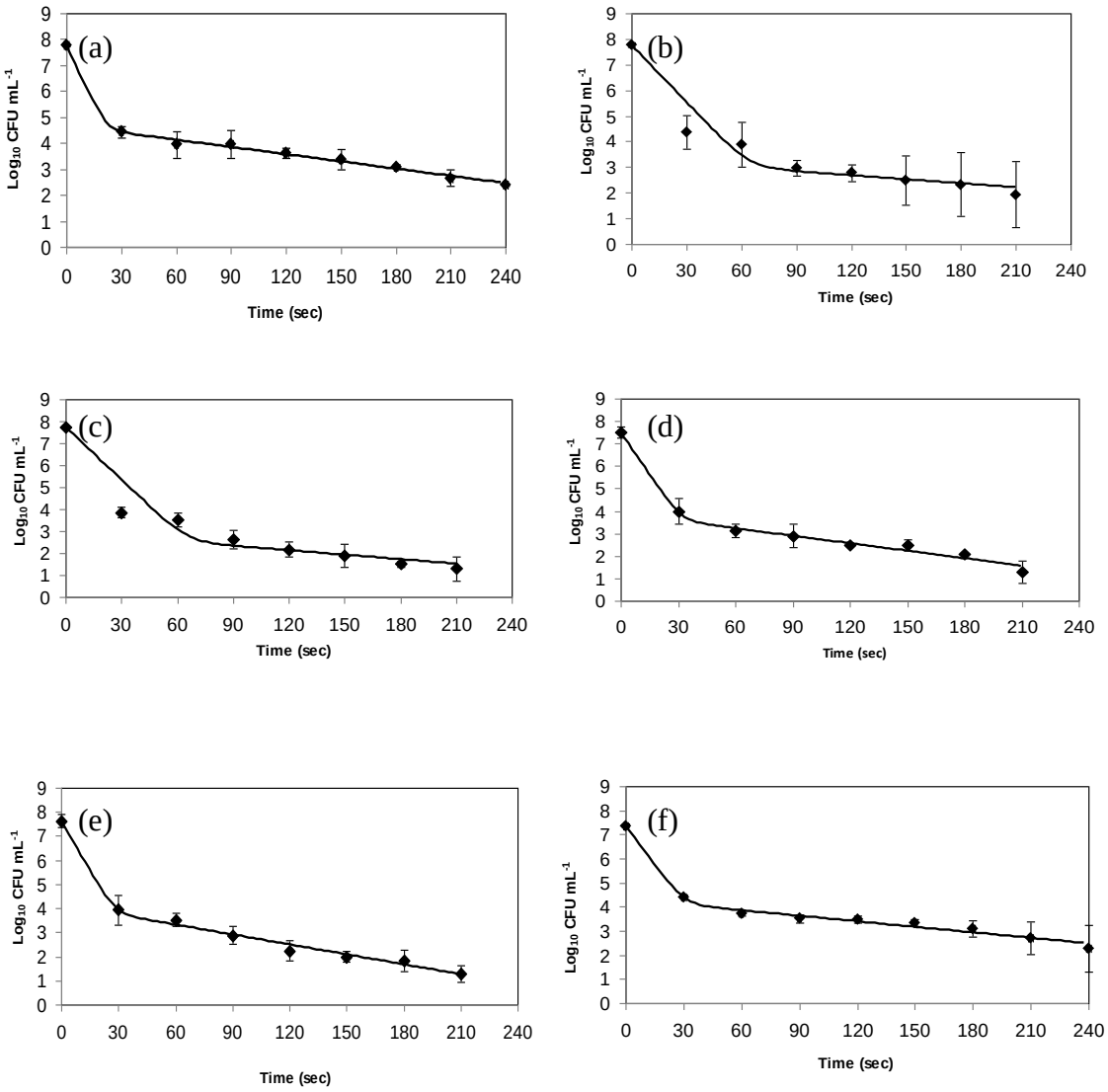
(a)
(b)
(c)
(d)
(e)
(f)
Log10 CFU mL-1
Time (sec)
0 30 60 90 120 150 180 210 240
0 1 2 3 4 5 6 7 8 9

Figure 1

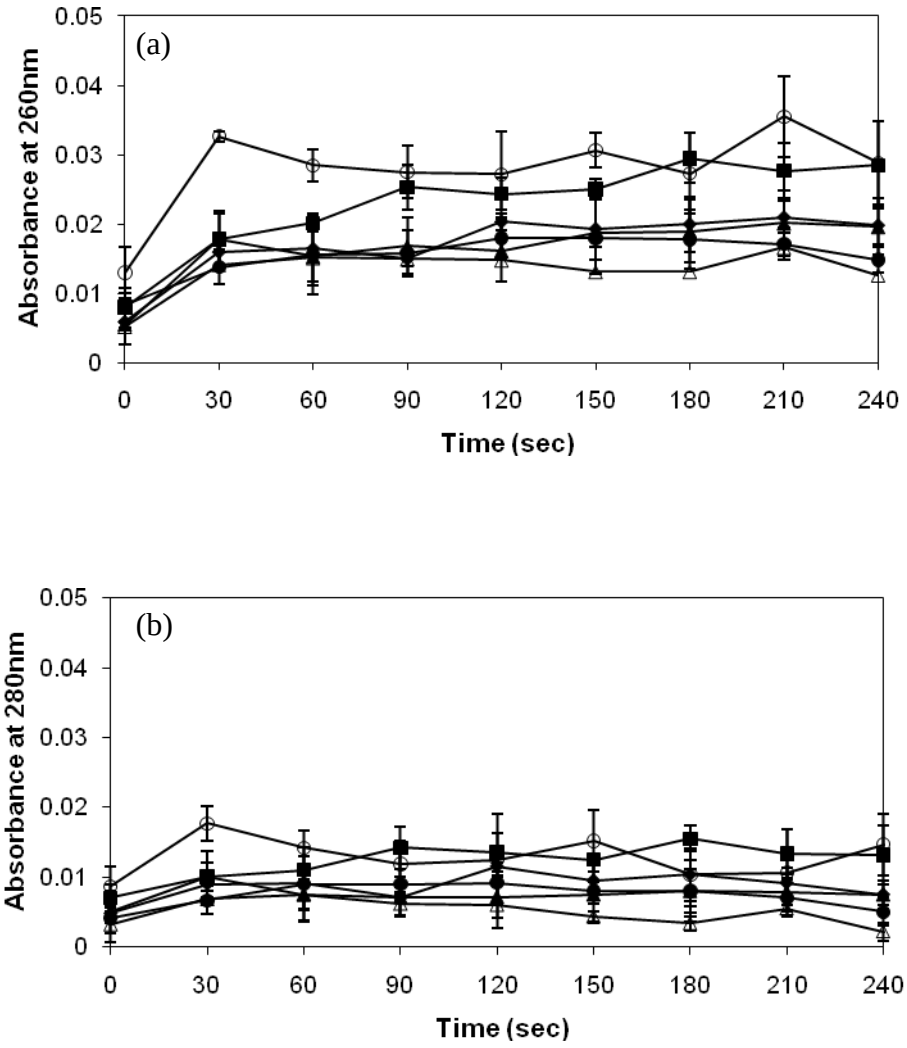
(a)
Absorbance at 260nm
0.05
0.04
0.03
0.02
0.01
0
0
30
60
90
120
150
180
210
240
Time (sec)
(b)
Absorbance at 280nm
0.05
0.04
0.03
0.02
0.01
0
0
30
60
90
120
150
180
210
240
Time (sec)

Figure 2

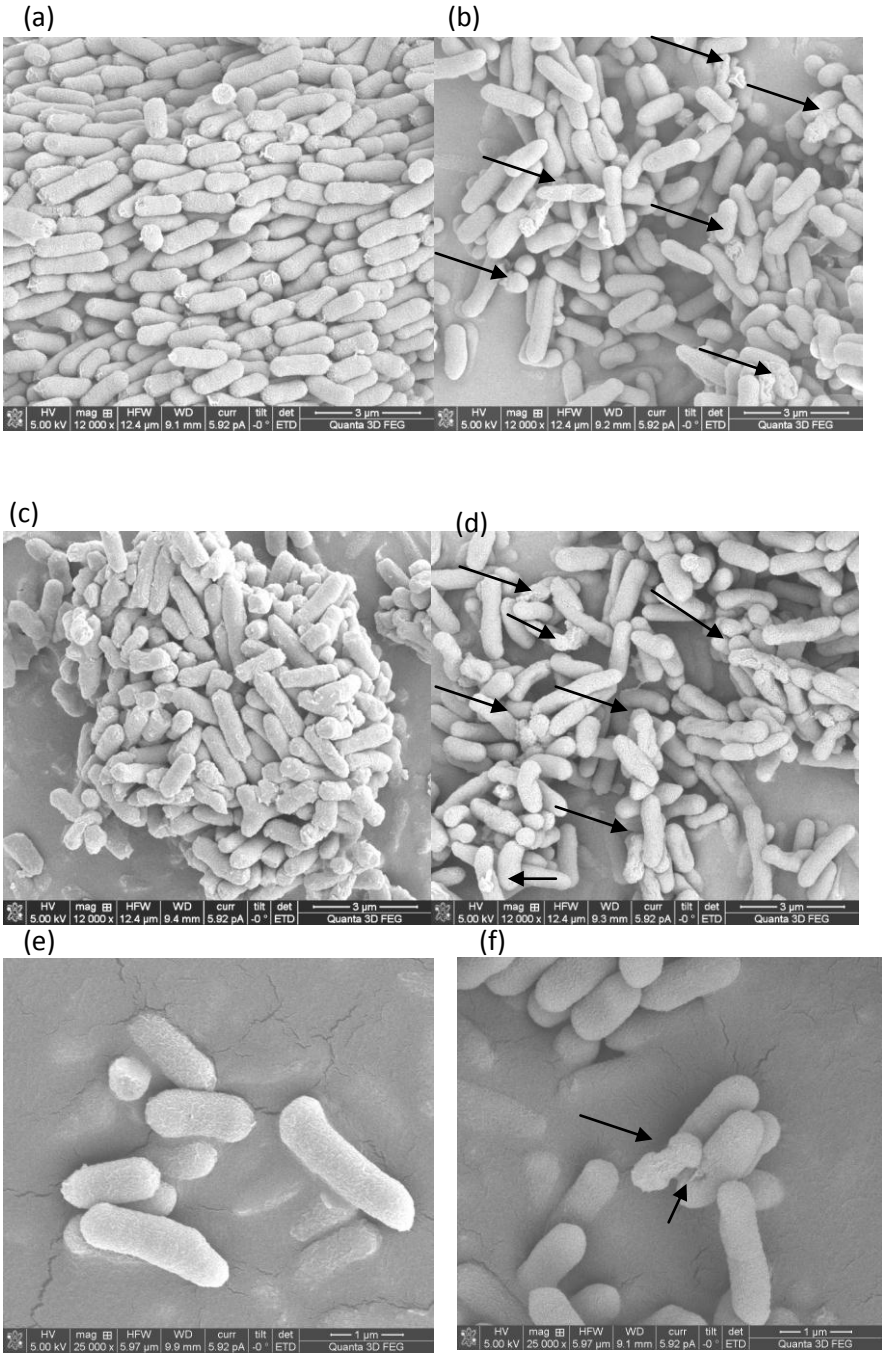

Figure 3