The Inactivation of *Bacillus subtilis* Spores at Low Concentrations of Hydrogen Peroxide Vapour

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Abstract

Spores of the bacterium *Bacillus subtilis* were deposited onto the surface of membranes by a process of filtration and exposed to concentrations of hydrogen peroxide vapour between 10 and 90 mg/m^3 (ppm) for times ranging from 1.5 to 48 h. The inactivation data obtained in this way was modelled using the Weibull, Series-Event and Baranyi inactivation models. The Weibull model provided the best fit, and its use was extended to previously published literature obtained at higher hydrogen peroxide concentrations to produce a correlation yielding *D* (decimal reduction value) values over a range from 10 to almost 4000 ppm.

Keywords: Hydrogen peroxide vapour, disinfection, *Bacillus subtilis* spores, inactivation kinetics, *D* values

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1. Introduction

Hydrogen peroxide possesses many properties that render it particularly useful as a sterilant and disinfectant; it is colourless and odourless and ultimately decomposes to water and oxygen. Hydrogen peroxide has been shown to inactivate a wide variety of infective biological agents ranging from both the vegetative cells and spores of bacteria and fungi (Rij and Forney, 1995; Rogers et al, 2005; Hall et al., 2008), protozoa and their cysts (Coulon et al, 2010), viruses (Pottage et al., 2010) and even prions (Fichet et al., 2007).

Although hydrogen peroxide can be applied either as a liquid or as a vapour for disinfection purposes, there is recent evidence to show that its mode of action in vapour form may be quite different from that in aqueous solution, and that the vapour is capable of bringing about more intensive oxidation of a range of biological macromolecules than do aqueous solutions of hydrogen peroxide (Finnegan et al., 2010). Interest in the use of hydrogen peroxide vapour to microbially decontaminate foods first arose some 20 years ago, and reasonably encouraging results were obtained for fruit products such as melons (Aharoni et al. 1994), grapes (Rij and Forney, 1995), prunes (Simmons et al., 1997), and apples (Sapers et al., 2003). One particular advantage of treating fresh produce is that the constituent plant catalases and peroxidases would act to breakdown any residual hydrogen peroxide at the surface of the produce harmlessly into water and oxygen (Vamosvigyazo,

1981). There has been a more sustained interest in using hydrogen peroxide vapour for decontaminating processing equipment such as freeze driers and centrifuges (Klapes and Vesley et al., 1990), aseptic filling machines (Kirchner et al., 2011) and food packaging (Pruss et al., 2012).

Previous studies on vapour hydrogen peroxide disinfection have been conducted at concentrations in the region of 1000 ppm (e.g. Wang and Toledo, 1986). In particular, there are no reports in the literature of the use of the hydrogen peroxide in vapour form at concentrations below 100 ppm. Establishing the efficacy of hydrogen peroxide vapour at low concentrations is of interest because in addition to proving lethal to a wide variety of micro-organisms, it is a powerful oxidising agent and thus able to corrode, or otherwise degrade, materials it comes into contact with (Maillard, 2011; Sk et al 2011) and therefore operating at relatively low hydrogen peroxide concentrations would serve to minimise damage to materials that were microbially contaminated.

One possible factor militating against wider use of hydrogen peroxide vapour disinfection is that previously published data has not been presented in a form that permits ready comparison either with other gaseous disinfectants such as chlorine dioxide, ozone etc., or indeed between different indicator organisms. A more rigorous approach to the design of such processes would entail the use of decimal reduction (D) values. In the work described here we investigated the inactivation of spores of the Gram positive bacterium *Bacillus subtilis* at hydrogen peroxide vapour concentrations in the range 10 to 90

ppm. We employed this particular organism because it is widely used in a variety of food-related inactivation studies such as UV light irradiation (Gardner and Shama, 1999) and high pressure treatment (Gao et al., 2007), and also because it is not a human pathogen. We modelled our inactivation data using the Weibull, Series-Events and Baranyi Inactivation expressions to arrive at decimal reduction values (D).

2 Materials and methods

2.1 Hydrogen Peroxide Exposure Chamber

Spores were exposed to hydrogen peroxide vapour in an environmental chamber that comprised a hydrogen peroxide vapour generation unit, an exhaust unit and three exposure boxes connected in series. Hydrogen peroxide solution of the required concentration was fed at a predetermined flow rate using a syringe infusion pump (WU-74900-05, Cole-Parmer Instrument Co., London). The solution was fed onto a hotplate maintained at a temperature of 130 °C. Upon evaporation the hydrogen peroxide vapour was mixed into the air flow generated by a fan (ACM150, Vent-Axia, Crawley, UK). From the vapour generation unit the air-hydrogen peroxide mixture flowed through a three-way valve into the first of the three Tecavinyl PVC exposure boxes. These were identical in construction, and the dimensions of each were height 100 mm; width 133 mm; length 665 mm. A reticulated foam gas flow distributor was placed at the entrance to each of the boxes to ensure good mixing of the air- hydrogen peroxide vapour mixture throughout the cross section of the box. Each box contained a sample support rack onto which membranes were placed at a height of 50 mm from the base of the box. The

lids of the boxes were secured and sealed in position by means of wing nuts; the lid of an individual box could be removed to withdraw samples without affecting the samples in the two other boxes. On exiting from the last of the three chambers, the air-hydrogen peroxide mixture flowed into the exhaust unit. The latter contained a hydrogen peroxide sensor (Model A11-34, ATI Ltd., Saddleworth, UK) and a combined humidity and temperature logger (Model OM-62, Omega Ltd., Manchester). The hydrogen peroxide sensor was calibrated before each experiment using a static equilibrated isothermal water bath containing a hydrogen peroxide/water mixture of known composition in a sealed vessel following the method of Frish et al., (2010). The saturated vapour pressure of hydrogen peroxide above the equilibrated mixture was obtained from published thermodynamic data. The sensors were calibrated over the concentration range 10-100 ppm. The establishment of steady-state conditions within the environmental chamber took approximately 2 hours. Spore-laden membranes were only introduced into the chamber after steady-state had been reached; the process of opening a box and placing the membranes within it did not cause undue disturbance of the peroxide concentration in the chamber which returned to the desired steady-state value within minutes following closure of the box.

Experiments were conducted at hydrogen peroxide concentrations of 10, 50, 75 and 90.

2.2 Deposition of spores of Bacillus subtilis on membranes

126 Spores of *Bacillus subtilis* (ATCC 6633) were prepared as described by
127 Harnulv and Syngg (1973). Spore stock was diluted to a concentration of 10^8
128 spores/ml and 1 ml of spore suspension, contained within a sterile
129 hypodermic syringe, was filtered through an Isopore™ membrane filter of 13
130 mm diameter and of 0.22 μm pore size (Millipore Ltd., Watford, UK) mounted
131 in a membrane filter holder, the whole assembly having been previously
132 sterilised by autoclaving. Following this, the filter holder was subjected to
133 gentle vacuum filtration at 0.5 barg in order to remove any liquid adhering to
134 the membrane. Membranes laden with uniformly deposited *B. subtilis* spores
135 were prepared for immediate use.
136
137 *2.3 Spore recovery and estimation of survival*
138 Following exposure to hydrogen peroxide the membranes were transferred to
139 sterile Universal bottles containing 10 ml phosphate buffered saline (PBS)
140 (Oxoid Ltd., Basingstoke, UK), 0.05 % (w/v) Tween 80 (Fisher Scientific,
141 Loughborough, UK) and 0.2 mg bovine liver catalase (2000-5000 Units/mg,
142 Sigma Chemical Co., UK). Catalase was added in order to arrest the action of
143 any hydrogen peroxide that had adsorbed on to the membranes; Johnston et
144 al. (2005) employed a similar approach. Membranes were then vortexed for 1
145 min and serially diluted as required in PBS before assessment of viability by
146 pour-plating into tryptone soya agar (TSA, Oxoid Ltd.,) in triplicate. Agar
147 plates were incubated at 37° C overnight before counting of controls. The
148 agar plates for peroxide exposed samples were incubated for a further 24
149 hours as the samples took longer to grow and the colonies were subsequently
150 counted.

151

152 *2.4 Scanning Electron Micrographs (SEM)*

153 The technique employed for fixing the spores to the membranes and their
154 subsequent drying for SEM was based on that of Perdigao et al. (1995) and
155 employed ethanol and hexamethyldisilazane for the latter stage. Samples
156 prepared by this method were then coated with a layer of gold-palladium and
157 then imaged using a Stereoscan 360 instrument (Cambridge Scientific
158 Instruments Ltd, Cambridge, UK) operated at 15 kV using a tungsten filament
159 at a working distance of 25mm.

160

161 *2.5 Inactivation Models*

162 Microbial inactivation data were fitted using three models: Baranyi, Series-
163 Event and Weibull. Fitting of experimental data was undertaken using Datafit
164 9.0 software (Oakdale Engineering, USA). We chose to indicate 'goodness of
165 fit' of all three models to the experimental data by recourse to the coefficient
166 of multiple determination (r^2). The data were weighted using $1/y_i^2$ where y_i
167 is the ordinate value of the i^{th} data point. Weighting the data in this way
168 permitted the fitting of experimental data over the entire log reduction range
169 without over-biasing fitting at low log reductions.

170

171 *2.5.1 Baranyi inactivation Model*

172 This mechanistic model was first proposed by Baranyi et al. (1996). The form
173 of the model used here was as follows:

174 $$\frac{C}{C_o} = \exp(-kt)\left\{\frac{1+\bar{C}}{1+\bar{C}\exp(-kt)}\right\} \quad (1)$$

where C_o is the initial bacterial spore concentration (number of spores per bioindicator), C bacterial spore concentration at any time t, *k* is the maximum inactivation rate and $\bar{C}$ is a dimensionless concentration of the hydrogen peroxide vapour (normalised by a Michaelis constant, K_c). Equation (1) has two fitting parameters (k and K_c), and these values were numerically optimised to achieve a best fit to a given set of experimental data. From a mechanistic point of view, the *k* parameter can be related directly to bacterial properties. The decimal reduction value *D* is defined as the time to reduce the concentration of viable spores to 10% of their starting value. *D*-values may therefore be calculated by manipulating eq.(1) to give the following form:

$$D = \frac{1}{k}\ln\left(10 + 9\bar{C}\right) \tag{2}$$

2.5.2 Weibull Model

The Weibull probability density function was originally formulated to predict the time-to-failure of mechanical components, but it has come to be widely applied to microbial inactivation by a variety of lethal agents. In this context, the model accounts for biological variation with respect to inactivation times. The following format of the model has been applied:

$$\frac{C}{C_o} = 10^{-\left(\frac{t}{D}\right)^p} \tag{3}$$

where the parameter *p* is commonly referred to as the ‘shape parameter’, and *D* is the decimal reduction value. The shape parameter accounts for upward

199 concavity of a survival curve ($p < 1$), a linear survival curve ($p = 1$), and
200 downward concavity ($p > 1$). Although the model is essentially of an empirical
201 nature, a link can be made with physiological effects. A value of $p < 1$
202 indicates that the remaining cells have the ability to adapt to the applied
203 stress, whereas $p > 1$ indicates that the remaining cells become increasingly
204 damaged. The Weibull model has two fitting parameters (D and p), these
205 values were optimised to achieve a best fit to the experimental data.
206
207 *2.5.3 Series-Event Model*
208 In this model an 'event' can be thought of as a 'quantum of damage' inflicted
209 on a living cell. A certain number of such events, occurring in series, need to
210 be accumulated by the cell for death to ensue. The modified series-event
211 model as described by Labas et al. (2008) with the following form of model
212 equation was used:
213

214 $$\frac{C}{C_o} = \left[\exp\left(-k\bar{C}^b t\right)\right]\sum_{i=0}^{n-1}\frac{\left(k\bar{C}^b t\right)^i}{i!} \quad (4)$$

215
216
217
218 The Series-Event model has three fitting parameters (rate constant k, reaction
219 order b and number of damaging events n), these values were numerically
220 varied to achieve a best fit to a given set of experimental data. The decimal
221 reduction, D value for the equation with the fitted parameters was obtained by

222 setting $C/C_o = 0.1$ in eq.(4) and finding the root of the function using Matlab
223 R2007b (Mathworks, USA).
224
225
226 **3 Results and discussion**
227
228 Operation of the environmental chamber for periods in excess of 48 hours
229 showed that the concentration of hydrogen peroxide remained constant. The
230 mean concentrations were 9.4 ppm (95% confidence interval (CI) 9.36-9.38)
231 over a period of 48 hrs, 50.8 ppm (95% CI 50.6-50.9), 73 ppm (95% CI 72.8-
232 73.1) and 93 ppm (95% CI 92.7-93.1) over a period of 6 hrs. As the relative
233 error of the sensor was 10 % of the nominal value, the concentrations quoted
234 throughout have been rounded for ease of reference.
235
236 The method of filtering spores of *B. subtilis* onto the membrane resulted in an
237 even surface distribution of the spores across the entire membrane (Figure 1).
238 Under such conditions all of the spores on the surface of the membrane would
239 have been equally exposed to environmental hydrogen peroxide. In previous
240 studies, commercially available 'spore strips' have frequently been used in
241 hydrogen peroxide vapour disinfection studies (e.g. Klapes & Vesley 1990;
242 Chung et al. 2008). These are typically produced by depositing the spore
243 suspension onto thin metal coupons and then allowing them to dry before
244 packaging them in a gas-permeable envelope. Their use was considered here
245 but visual examination of the coupons produced by two different
246 manufacturers showed that the dried spores were not evenly deposited on the

surface of the metal. This was indicative of spore stacking and the potential for the establishment of diffusional resistances which would result in the spores not being uniformly exposed to the hydrogen peroxide vapour. A comparison of surface distribution of bacteria on membranes (by SEM), obtained by either filtration (as used here) or direct pipetting followed by drying in air, confirmed that a highly heterogeneous distribution was obtained in the latter case (Bayliss et al., 2012).

Figure 2 shows the results obtained for exposure of *B. subtilis* spores at hydrogen peroxide concentrations of 50, 75 and 90 ppm for times up to 6 h with all experiments being carried out in duplicate (averages are shown). The protocols described under § 2.4 resulted in a consistent recovery of 50 % of the spores applied to the membranes and the data plotted in Figure 2 were corrected accordingly. At a concentration of 90 ppm, 6.5 log reductions were obtained after 6 h, whilst at 50 ppm only 1.4 log reductions were achieved over the same period. Results for exposure of the spores to a lower hydrogen peroxide concentration of 10 ppm for contact times up to 48 h are not shown — significantly longer contact times were needed to achieve a 1 log reduction (in excess of 36 h). The form of the inactivation curve for the 10 ppm data was a similar shape to that obtained in Figure 2 for higher hydrogen peroxide concentrations.

The results obtained by fitting these data to the three inactivation models — the Series-Event Model, the Baranyi and the Weibull — are shown in Table 1. The table shows the fitting parameters for each model, the goodness of fit (r^2)

and an estimate of the decimal reduction value (*D*). In the case of the Series-Event model the best fit to the data was obtained for the case where the susceptible target has to be hit a total of 7 times to bring about inactivation of the *B. subtilis* spores. The *D* values predicted by each model showed reasonable agreement at all three concentrations of hydrogen peroxide. However, the highest values for r^2 were obtained using the Weibull model and Figure 2 depicts the fit of this model to the data.

Wang and Toledo (1986) had earlier examined the inactivation of *B. subtilis* spores by hydrogen peroxide vapour at concentrations in the range 275 to 3879 ppm, which is much higher than those employed here. However, they had not attempted the derivation of *D* values from their data. Figure 3 shows inactivation data for *B. subtilis* obtained from literature (Wang and Toledo, 1986). The three inactivation models employed in this work were also applied to these data and the Weibull model provided the highest values of r^2 for the entire data set (see Table 2).

In Figure 4 *D* values calculated using the Weibull model are plotted against hydrogen peroxide vapour concentration over the range 10 to 4000 ppm encompassing both our data along with that of Wang and Toledo (1986). A power-law regression model describes the hydrogen peroxide concentration dependency of the decimal reduction values both for both data sets. Interestingly Wang and Toledo (1986) did not employ commercial spore strips in their study, but instead deposited spores of *B. subtilis* on various types of packaging materials. The compatibility with our data suggests that the

297 condition of the spores on the surface of these materials must have been
298 similar to ours as depicted in Figure 1.
299
300 Johnston et al. (2005) obtained linear inactivation kinetics (log_{10} survivors vs.
301 time) for *C. difficile* spores, employing exposure times of less than 10 min, but
302 at concentrations much higher than those used here (a maximum hydrogen
303 peroxide concentration of 355 ppm). In a study of inactivation of a variety of
304 nosocomial bacteria, the data presented by Otter and French (2009)
305 displayed the same characteristics as shown in Figure 2. The shapes of the
306 inactivation curves obtained here were also similar to those shown by Pottage
307 et al. (2010) for MS2 bacteriophage.
308
309 Surprisingly, not all previous workers who have conducted microbial
310 inactivation studies with hydrogen peroxide have provided full details of the
311 concentration of the oxidant over the time course of their experiments.
312 Moreover in certain cases the concentration of hydrogen peroxide has not
313 remained constant over time, which hinders application of the data. Chung et
314 al. (2008) provided a hydrogen peroxide concentration-time curve for their
315 work on the inactivation of *Geobacillus stearothermophilus* spores, whereas,
316 Johnston et al. (2005) and Hall et al. (2008) specified only the peak hydrogen
317 peroxide concentrations, and Pottage et al. (2010) published no hydrogen
318 peroxide concentrations whatsoever. The absence of full concentration-time
319 information prevented estimates of *D* values from being made and compared
320 with those for *B. subtilis* spores.
321

4 Conclusions

The work described here enabled estimates to be made of *D* values for spores of *B. subtilis* over a wide range of hydrogen peroxide concentrations. This should enable decontamination processes based on this particular oxidant to be rigorously designed and would lend confidence to the predictions obtained. Although the *D* values at the lowest concentrations of hydrogen peroxide employed here (10 ppm) are of the order of 10^3 minutes, bacterial spores are considerably more resistant to oxidative treatments than are vegetative bacteria, and therefore relatively low concentrations of hydrogen peroxide may still be able to bring about significant reductions in the viability of food-related pathogens that do not form spores such as *Listeria*, *Salmonella* and *Campylobacter*. Otter and French (2009) reported that following the release of a fixed quantity of hydrogen peroxide into an enclosed space, the environmental concentration of the oxidant dropped rapidly as mixing occurred, and therefore it is important to be able to account for the disinfective effect of hydrogen peroxide under such conditions.

Food processing environments are frequently colonised by pathogenic bacteria that are able to persist in those environments despite the frequent application of liquid disinfectants (Chambel et al., 2007). One reason for the persistence of such organisms may be that they might evade inactivation by becoming lodged in locations where liquids might not be able to gain access to. A recent study has shown that hydrogen peroxide vapour is particularly effective at decontaminating complex and intricate three dimensional shapes

that liquid disinfectants would have difficulty accessing (Unger-Bimczok et al., 2011). Therefore the use of hydrogen peroxide vapour may offer the possibility of eliminating persistent pathogens from both food processing equipment and environments in a manner analogous to that currently being employed in healthcare environments for the inactivation of nosocomial pathogens (Otter and French, 2009).

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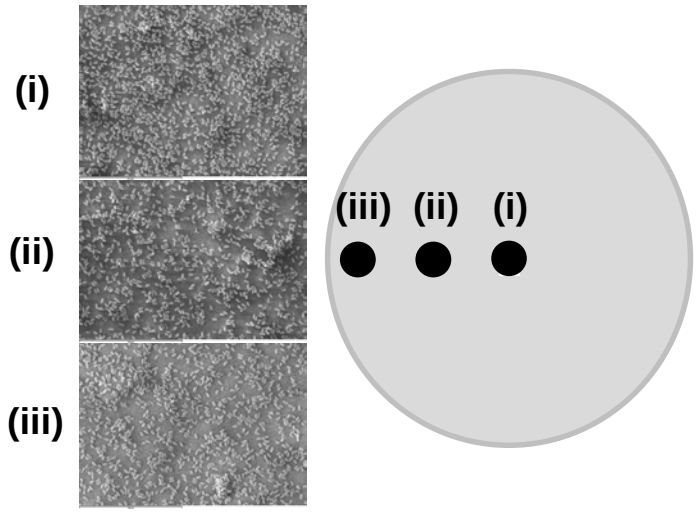

Figure 1. SEM images of *B. subtilis* spores deposited onto a filter membrane at 9000 times magnification. In each case the area sampled has dimensions of 67 µm x 50 µm. (i) centre, (ii) intermediate, (iii) edge.

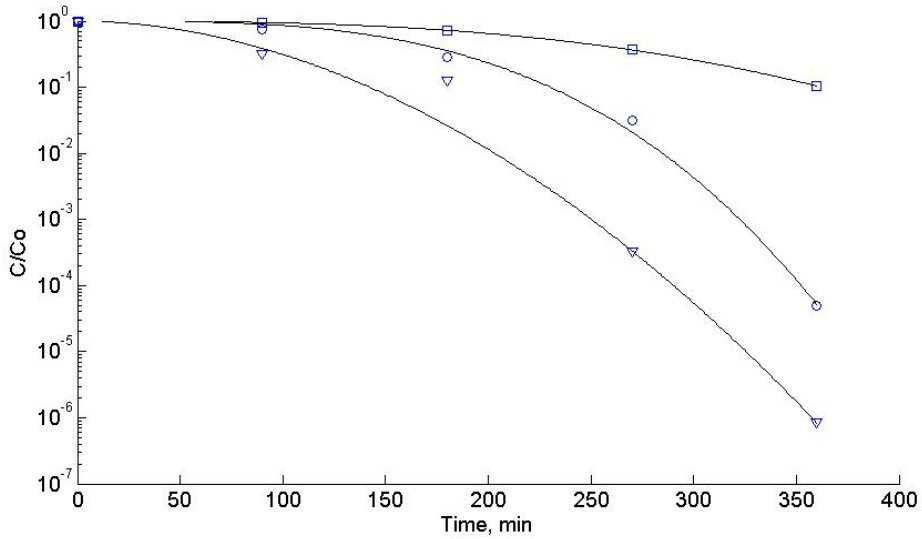

Figure 2. *B. subtilis* spore inactivation data (solid lines are data fits using Weibull regression model), squares (50ppm); circles (75ppm); triangles (90ppm).

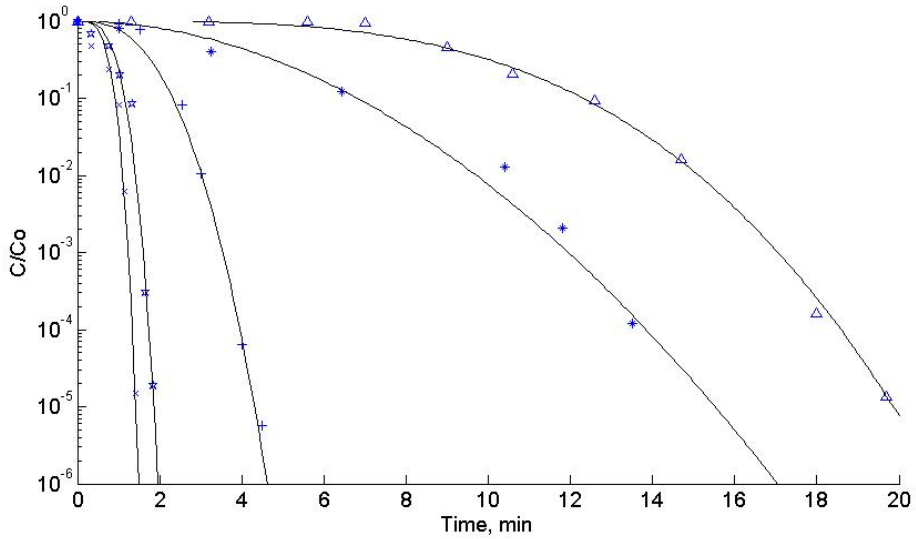

Figure 3. *B. subtilis* spore inactivation data (solid lines are data fits using Weibull regression model), triangles (275ppm); asterisk (558ppm); plus (1131ppm); star (1859ppm); x (3879ppm).

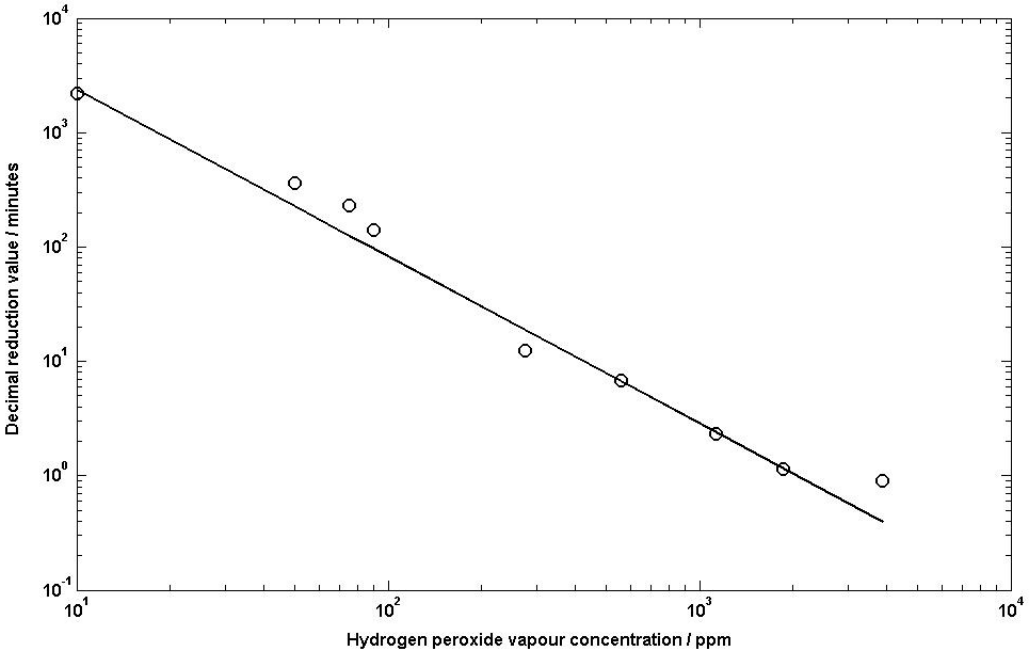

Figure 4. Decimal reduction values for spores of *B. subtilis* as a function of hydrogen peroxide vapour concentration, (solid line is a power law fit, $D = kC^m$, where k = 69451, m = -1.462, $r^2 = 0.817$).

485 Table 1. *B. subtilis* inactivation kinetics (10-90ppm) modelling parameters and
486 decimal reduction values.
487

	Baranyi				**Weibull**			**Series-Event**			
ppm	**k**	**K_c**	**r^2**	**D**	**P**	**D**	**r^2**	**B**	**k**	**r^2**	**D**
10	2.70E-03	3.31E-01	0.888	2090	2.36	2186	0.913	4.29E-01	2.03E-03	0.864	2159
50	1.75E-02	8.13E-01	0.999	362	2.82	362	0.999	2.17E-01	1.39E-02	0.996	362
75	5.00E-02	2.10E-02	0.797	208	3.25	230	0.948	2.22E-01	2.53E-02	0.683	178
90	4.90E-02	2.04E-00	0.621	123	1.94	142	0.829	2.27E-01	2.93E-02	0.648	145

Table 2. *B. subtilis* inactivation kinetics (275-3879 ppm, data obtained from literature) modelling parameters and decimal reduction values.

	Baranyi				**Weibull**			**Series-Event**			
ppm	**k**	**K_c**	**r^2**	**D**	**P**	**D**	**r^2**	**B**	**k**	**r^2**	**D**
275	1.1	1.0E-02	0.864	11.3	3.36	12.3	0.981	-5.89E-03	1.33E+00	0.611	9.2
558	0.9	3.0E+01	0.729	5.8	1.95	6.8	0.852	5.50E-02	1.18E+00	0.660	7.0
1131	5.0	3.8E-02	0.990	2.5	2.60	2.3	0.880	1.01E-01	2.90E+00	0.740	2.0
1859	10.9	1.4E-01	0.678	1.1	3.33	1.2	0.864	1.03E-01	6.24E+00	0.562	0.9
3879	15.5	8.1E-02	0.436	0.8	3.62	0.9	0.714	1.15E-01	7.02E+00	0.423	0.7

Research Highlights

- *B. subtilis* spores were exposed to low concentrations of H_2O_2 vapour
- D values were derived by mathematically modelling the inactivation kinetics.
- Best fits of our data were obtained using the Weibull model
- D values are quoted for the range10 to 4000 ppm H_2O_2