Application of response surface methodology for modeling and optimization of *Bacillus subtilis* spores inactivation by the UV/persulfate process

Zahra Sabeti, Mahmood Alimohammadi, Samira Yousefzadeh, Hassan Aslani, Maryam Ghani and Ramin Nabizadeh

ABSTRACT

Stronger disinfection techniques are required to inactivate *Bacillus subtilis* spores as surrogate microorganisms for *Cryptosporidium parvum* oocysts. In this study, the effects of UV and persulfate separately and also in combination were investigated on *B. subtilis* spore inactivation. Central composite design and response surface methodology were used to optimize target microorganism reduction. Contact time, initial pH, and persulfate dosage were considered as input experimental variables. Based on the design of the experiments, first and second order response surface models have been developed to correspond to the output response of *B. subtilis* spore reduction. It can be concluded that microbial reduction by UV alone was more effective than persulfate, while the combined UV/persulfate process demonstrated the highest log reduction (4.1) under the following optimal conditions: 60 min contact time, pH = 7.8, and persulfate dosage of 30 mM. On the other hand, the optimal condition for UV treatment was a contact time of 60 min at a pH of 5.0, which led to a 3.19 log spore inactivation. Consequently, the UV/persulfate system can be introduced as an alternative disinfectant for the inactivation of *B. subtilis* spores.

Key words | *Bacillus subtilis* spores, disinfection, persulfate, response surface methodology, UV radiation

Zahra Sabeti
Mahmood Alimohammadi
Samira Yousefzadeh
Hassan Aslani
Maryam Ghani
Ramin Nabizadeh (corresponding author)
Department of Environmental Health Engineering,
School of Public Health,
Tehran,
Iran
E-mail: *rnabizadeh@tums.ac.ir*

Hassan Aslani
Department of Environmental Health Engineering,
School of Health, Tabriz University of Medical Sciences,
Tabriz,
Iran

Ramin Nabizadeh
Center for Air Pollution Research (CAPR),
Institute of Environmental Research (IER), Tehran University of Medical Sciences,
Tehran,
Iran
and
Tehran University of Medical Sciences,
Floor Number 5, Ghods Avenue, Enghelab Street, Enghelab Square,
Tehran,
Iran

INTRODUCTION

Disinfection is a very crucial step in water treatment processes for destroying pathogenic microorganisms (Post *et al.* 2011). Traditionally, chlorination has been the most commonly adopted disinfection process for the treatment of drinking water. However, with the appearance of chlorine-resistant pathogenic microorganisms such as *Giardia lamblia* cysts and *Cryptosporidium parvum* oocysts, many conventional drinking water treatment plants that use free chlorine are faced with problems in reaching the required target goals for microbial inactivation (Jung *et al.* 2008). Since the direct measurement of *C. parvum* or *G. lamblia* is difficult, time-consuming and expensive, *Bacillus subtilis* spores were selected as an indicator (Facile *et al.* 2000; Driedger *et al.* 2001).

Bacillus subtilis is an aerobic, non-pathogenic, gram-positive soil bacterium that is easy to count and identify in laboratory conditions. Under certain conditions (e.g., starvation), spores are formed that are highly resistant to disinfection (Driedger *et al.* 2001; Radziminski *et al.* 2002). It has been reported that the *Bacillus* spores are 10–75 times more resistant than its vegetative form (Blatchley *et al.* 2005).

Due to several disadvantages associated with chlorination, current studies have extensively investigated alternative disinfection processes such as ozone, UV

doi: 10.2166/ws.2016.139

irradiation, chlorine dioxide, chloramines, plasma discharge and electrochemical processes as well as the sequential or combined processes for the removal of resistant microorganisms (Radziminski *et al.* 2002; Larson & Mariñas 2003; Cho *et al.* 2006; Jung *et al.* 2008, 2010; Mezule *et al.* 2014). Today most of the advanced oxidation process studies in water and wastewater disinfection have focused on hydroxyl radicals ($OH^{\cdot}$); however, only a few studies discuss other radical species. For example, sulfate radicals ($SO_4^{\cdot-}$) have a significantly high reduction potential of 2.5–3.1 V, and that of $OH^{\cdot}$ is 1.9–2.9 V (Lin *et al.* 2011; Gao *et al.* 2012; Wordofa 2014). Moreover, $SO_4^{\cdot-}$ also exhibits a nonselective oxidation pattern, and is capable of quickly decomposing most of the organic pollutants and biological toxins in water (Lin *et al.* 2011; Gao *et al.* 2012; Tan *et al.* 2013; Xie *et al.* 2015). $SO_4^{\cdot-}$ is produced via activation of peroxydisulfate ($S_2O_8^{2-}$, commonly known as persulfate) with UV, catalysts (e.g., Mn^{2+}, Fe^{2+}), heat and high pH (Roshani & Leitner 2011; Gao *et al.* 2012; Tan *et al.* 2013; Lutze *et al.* 2015; Xie *et al.* 2015). Among these methods, UV-based oxidation has a high potential destruction rate due to the production of more powerful components such as sulfate radicals (Tan *et al.* 2013). Nevertheless, there is insufficient information about the application of sulfate radicals in water disinfection processes and especially for inactivating *B. subtilis* spores.

Response surface methodology (RSM) is a compilation of statistical and mathematical techniques valuable for developing, improving, and optimizing processes (Carley *et al.* 2004). Prior to applying the RSM, an experimental design was implemented to determine which experiments should be carried out in the experimental region being studied (Bezerra *et al.* 2008). RSM was used to model and optimize spore reduction (Huang *et al.* 2007). In this study, a central composite design (CCD) was used as a novel approach to optimize the log reduction of *B. subtilis* spores by the UV/persulfate process. Contact time, pH, and concentration of persulfate solution were studied as key parameters affecting disinfection in this investigation.

MATERIALS AND METHODS

The materials used in this study included sodium persulfate (Merck, No. 106609), NaOH (Merck, No. 105587), H_2SO_4 (Merck, No. 100731), sodium thiosulfate (Merck, No. 106512) and NaCl (Merck, No. 106406). Necessary stock solutions were made from the above-mentioned neat materials and double distilled water. All glassware was washed using tap water and rinsed with distilled water. All of the instruments were sterilized by autoclaving at 121 °C for 15 min prior to use.

Spore production and sample preparation

Bacillus subtilis (ATCC 6633) vegetative cells were cultured in tryptic soy agar (TSA, Merck No. 105458) medium and incubated for 24 h at 35 °C. *In vitro* production of *B. subtilis* spores was conducted by cultivating the vegetative cells at 37 °C on the antibiotic assay medium 1 (Himedia, M003) supplemented by 0.3 g L^{-1} $MnSO_4$ for 5–7 days (Radziminski *et al.* 2002). Subsequently, the spore suspension was washed from the top of the medium by saline water and transferred to 10 mL sterile centrifuge tubes. The suspension was centrifuged for 15 min at 4,000 *g* and stored in a 4 °C refrigerator until use.

For sample preparation, 4 mL of the spore suspension was poured into 500 mL of sterilized drinking water. For further inactivation of vegetative cells, the samples were heated up to 80 °C and kept for 12 min at this temperature (Larson & Mariñas 2003). The turbidity of this suspension was between 25 and 32 Nephelometric Turbidity Units (NTU), which led to the production of 10^6–10^8 spores/mL.

Experimental design

The CCD was employed to investigate the process of *B. subtilis* inactivation using UV, persulfate, and the combination of UV/persulfate. The RSM was used to determine the combined effects of contact time (X_1), pH (X_2), and persulfate dosage (X_3) on log inactivation of *B. subtilis* (Y) as the output response. The study was divided into three different phases including the UV, persulfate, and combined UV/persulfate phases. In the first phase, 17 experiments were designed to determine the UV radiation effect on *B. subtilis* inactivation. In the second phase, a design considering the three above-mentioned controllable variables was carried out. An orthogonal CCD in two cube and star blocks was designed. A total of 26 experiments were carried

out consisting of 12 center points, $2^3 = 8$ design points, and $2 \times 3 = 6$ axial points. The design of the third phase was similar to that of the second phase except for including a constant UV irradiation in the last phase. *Bacillus subtilis* spore removal served (Y) as the dependent variable of the process. The actual and coded levels of the design are shown in Table 1. The design, mathematical modeling, and optimization of this work were implemented by the RSM package (Lenth 2009). There are two important models in the RSM: first order and second order model. A general explanation of the first order model and the quadratic model is based on Equations (1) and (2), respectively (Carley *et al.* 2004; Khuri & Mukhopadhyay 2010).

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \varepsilon \quad (1)$$

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \varepsilon \quad (2)$$

where y is the response; X_i and X_j are the variables; β_0 is the constant coefficient; β_i, β_j, β_{ij} are the interaction coefficients of linear, quadratic, and second order terms, respectively; k is the number of studied parameters; and ε is the system error.

The effect of each factor was evaluated by analysis of variance (ANOVA) (95 and 99%). The model permitted the evaluation of the effects of linear, quadratic and interactive terms of the independent variables on the dependent variable. The response surface and contour plots were drawn to illustrate the main and interactive effects of the independent variables on disinfection efficiency. The optimum values of the selected variables were obtained by solving the regression equation and by analyzing the response surface plot.

Table 1 | Coded and real values of the Independent variables used

		Range and levels				
Variables	**Symbol**	$-\propto\sqrt{2}$	**−1**	**0**	**1**	$+\propto\sqrt{2}$
Contact time (min)	X_1	5	16.1	32.5	48.8	60
pH	X_2	5	6.2	8	9.7	11
Concentration of persulfate (mM)	X_3	3	8.4	16.5	24.5	30

Experimental procedure

Disinfection experiments for the three candidate disinfection processes (persulfate alone, UV alone and combined UV/persulfate process) were performed at different contact times (5–60 min) and pH ranges of 5–11. It should be noted that a contact time exceeding 60 min is not convenient in the disinfection process. In addition, the range of the initial pH was chosen according to previous literatures (Lin *et al.* 2011; Tan *et al.* 2013; Wordofa 2014; Xie *et al.* 2015). The persulfate concentration was also varied from 3 to 30 mM, and the minimum persulfate dosage was chosen based on a study of the effects of sulfate radicals on the reduction of *E. coli* (Wordofa 2014), while the maximum amount was selected according to the fact that the spores are more resistant microorganisms.

In order to estimate the enhancement resulting from the combined process, inactivation efficiencies were compared with those of individual processes. Both the UV and UV/persulfate processes were performed using a low pressure UV lamp, which emitted monochromatic UV radiation at 254 nm. The UV intensity was measured as 112 μW/cm^2 at the distance of 125 mm between the lamp and water surface. The dose can be calculated by multiplying the mentioned intensity by the contact times and is presented as mW.s/cm^2.

Fifty milliliters of synthetically prepared sample was poured into a sterile petri dish (90 mm diameter), and the solution's pH was adjusted to the desired value using a few drops of H_2SO_4 or NaOH. In the UV experiments, the sample was put under a UV lamp and gently stirred throughout the exposure period. In the case of the persulfate and UV/persulfate procedures, the persulfate stock solution was prepared in such a way that only 1 mL was required to provide the defined persulfate concentration. The sample was gently stirred throughout the contact time. At the end of each contact time, residual persulfate was neutralized by adding 1 mL of sodium thiosulfate based on a mole to mole ratio. At the end of each exposure, 10 mL of the disinfected sample was collected for microbial analysis.

Microbiological analysis

Viability was determined by the membrane filtration technique using nitrocellulose filters (Sartorius Stedim, No.

11406) with a 47 mm diameter and a nominal pore size of 0.45 μm (Larson & Mariñas 2003). For each sample, a dilution series was made by transferring 10 mL of sample to a 100 mL dilution bottle containing 90 mL of 9% saline water. After filtration, the filters were placed in a petri dish containing 5–6 mL of nutrient agar (Merck, No. 1.05450) and 0.015 mg L^{-1} of trypan blue (Titrachem, No. 512). Colonies were counted after incubation at 37 °C for 24 h (Larson & Mariñas 2003).

RESULTS AND DISCUSSION

CCD and regression models

In this study, the effects of contact time, pH, and persulfate dosage were investigated on *B. subtilis* spore inactivation separately, and also in combination with UV and persulfate. The experiments were performed using a CCD, and the corresponding results for the UV and UV/persulfate processes are shown in Tables 2 and 3, respectively. Multiple regression analysis of three experimental phases is presented in Table 4. Multiple R^2, adjusted R^2, and *p*-value were used to assess the model adequacy. Both contact time and pH were significant in the UV process alone, while no interaction was observed between the variables (Table 4). It should be mentioned that terms which could show interactions were omitted from the final model to optimize the model, since these were not statistically meaningful.

The difference between R^2 and R^2_{adj}, less than 0.2, as well as a lower *p*-value (1.56×10^{-8}), suggests that the model is reliable. All of the linear coefficients in the persulfate process were not significant (Table 4). A lower value of model R^2 and significant difference of more than 0.2 between R^2

Table 2 | Arrangement of the CCD for the two independent variables used in the UV process

Run no.	Exposure time (X_1)	pH (X_2)	Log inactivation (Y)
1	32.5	8	1.448158
2	13	10	0.062148
3	32.5	8	1.477121
4	13	5.8	0.029963
5	52	10	1.833669
6	52	5.8	2.435729
7	32.5	8	1.462398
8	32.5	8	1.492361
9	32.5	8	1.246672
10	32.5	11	1.176091
11	5	8	0
12	32.5	8	1.477121
13	32.5	8	1.455932
14	32.5	5	1.778151
15	60	8	3.259637
16	32.5	8	1.641431
17	32.5	8	1.632023

Table 3 | Arrangement of the CCD for the three independent variables in the UV/persulfate process

Run no.	Contact time (X_1)	pH (X_2)	Persulfate concentration (X_3)	Log inactivation (Y)	
				Actual	Predicted
1	16	6	24.5		
2	49	6	24.5	2.30103	2.277813
3	16	10	8.5	0.330993	0.784313
4	32.5	8	16.5	1.315753	1.118263
5	32.5	8	16.5	0.90309	1.118263
6	16	10	24.5	1.346787	1.577113
7	32.5	8	16.5	0.989276	1.118263
8	32.5	8	16.5	0.929962	1.118263
9	16	6	8.5	0.167491	0.867913
10	49	10	8.5	1.69897	1.401413
11	32.5	8	16.5	1.610834	1.118263
12	49	10	24.5	1.69897	2.194213
13	49	6	8.5	1.802719	1.485013
14	32.5	8	16.5	1.022276	1.118263
15	32.5	8	16.5	1.014723	1.118263
16	32.5	8	16.5	1.875061	1.118263
17	32.5	8	30	4.051153	3.58235
18	32.5	8	3	2.363178	2.2445
19	32.5	8	16.5	0.937852	0.571288
20	32.5	8	16.5	1.034762	1.118263
21	32.5	8	16.5	0.967815	1.118263
22	32.5	5	16.5	1.034762	0.691363
23	60	8	16.5	0.937852	1.632513
24	32.5	8	16.5	1.210853	1.118263
25	32.5	11	16.5	0.967815	0.565963
26	5	8	16.5	0.69897	0.604013

Table 4 | Regression coefficients and their significance

Model term	Coefficient estimate	Standard error	*t* value	*p*(>|t|)
UV				
Intercept	0.216537	0.358525	0.604	0.5555
Time	0.056403	0.004398	12.824	3.97e-09***
pH	−0.080641	0.040558	−1.988	0.0667
R^2: 0.9233, Adjusted R^2: 0.9123, F-statistic: 84.21 on 2 and 14 DF, *p*-value: 1.568e-08				
Persulfate				
Intercept	0.047522	0.446634	0.106	0.916
pH	0.041435	0.046474	0.892	0.382
Time	−0.003002	0.004892	−0.614	0.546
Per. concentration	−0.002442	0.011100	−0.220	0.828
R^2: 0.05254, Adjusted R^2: −0.07666, F-statistic: 0.4067 on 3 and 22 DF, *p*-value: 0.7497				
Persulfate/UV				
Intercept	1.118091	0.112403	9.9471	0.459e-09***
Time	0.514252	0.172432	2.9824	0.007362**
pH	−0.062135	0.178168	−0.3487	0.730928
Per. concentration	0.667458	0.191492	3.4856	0.002332**
I (pH^2)	−0.489495	0. 273205	−1.7917	0.088330
I (Per. concentration^2)	1.794601	0.291788	6.15045	0.215e-06***
F-statistic: 12.39, on 5 and 20 DF *p*-value: 1.456e-05				

**$p<0.01$, ***$p<0.001$.

and R^2_{adj} shows that the model is not significant and acceptable; moreover, the higher *p*-value of 0.749 implies that there is a weak correlation between the variables and the response produced by the model.

The results of the reduced quadratic model for the UV/persulfate process are also shown in Table 4. All linear coefficients, except pH, and two quadratic terms (X_2^2 and X_3^2), were significant ($p < 0.05$), while no significant interaction was found ($p > 0.05$). In addition, the model R^2 is promising and it is very consistent with R^2_{adj} (Table 5). Statistical testing of the model was done in the form of ANOVA analysis, which is required to test the significance and adequacy of the model. A summary of ANOVA results for the selected quadratic model for the UV/persulfate process is provided in Table 5. The higher *F* value of 19.64 and the lower probability value of $p < <0.05$ indicates that the model is highly significant. The model also indicates a statistically insignificant lack of fit.

Response surface and contour plotting

The graphical representations of the regression models, called the response surfaces and the contour plots, were obtained using the R software (Figures 1 and 2). In the

Table 5 | ANOVA for the developed RSM model for the UV/persulfate process

Source	R^2	Adjusted R^2	Df	Sum sq	Mean sq	*F* value	Pr > *F*
Model	0.756	0.695	2	6.9288	3.4644	19.6445	>0.00001
Residuals	–	–	20	3.5271	0.1764	–	–
Lack of fit	–	–	10	2.5461	0.2546	2.5955	0.074224
Pure error	–	–	10	0.9810	0.0981	–	–

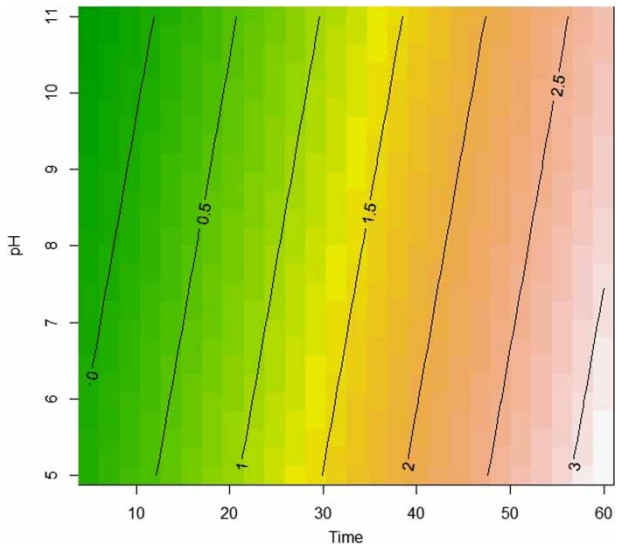

Figure 1 | Contour plot of the effect of pH and exposure time on log inactivation of *Bacillus subtilis* spores by UV irradiation.

case of the UV process, it is clear that the spore reduction increased in a pH range of 5 and 11 from less than 0.5 to 3 log in a 5 to 60 min duration (Figure 1). The results obtained for inactivating *B. subtilis* spores (less than 1 log) with persulfate alone in conditions used in this study (i.e., time: 5–60 min, pH: 5–11, and persulfate concentration 3–30 mM) were not promising.

The results (Table 4 and Figure 1) show that the most important factor affecting disinfection efficiency in the UV alone process was exposure time, and the reduction pattern was in good consistency with the results reported in the literature (Cho *et al.* 2006). According to Cho *et al.* (Cho *et al.* 2006), pH did not affect spore inactivation in the UV_{254} process, and a 2 log reduction was achieved at both pH 5.6 and 8.2. Moreover, Mamane *et al.* (Mamane *et al.* 2007) did not obtain any spore inactivation at higher wavelengths (>295 nm).

The next step was to investigate the effect of activated persulfate by UV irradiation on *B. subtilis* spore inactivation. The perspective plot illustrates how the microbial reduction varied with the independent variables in the combined UV/persulfate process (Figure 2). *Bacillus subtilis* spore reduction as a function of persulfate concentration and pH at a fixed time (31.38 min) is shown in Figure 2(a). A higher spore reduction was achieved when the persulfate concentration increased from 25 to 30 mM and the pH varied from 7 to 9. Figure 2(b) shows the effect of persulfate concentration and contact time at a fixed pH of 8. The reduction of *B. subtilis* spores in the persulfate dosage range of 23 to 30 mM increased from 2 to 4 log as a function of increased time. The effect of pH and time sliced at 16.5 mM persulfate dosage are displayed in Figure 2(c). The reduction of *B. subtilis* spores increased with time at a slightly alkaline pH (i.e., 7–9), reaching a 1.5 log reduction after 30–60 minutes of contact time.

The persulfate dosage can significantly affect disinfection efficiency in this process because higher concentrations lead to more sulfate radical generation. Our results, in line with other studies (Tan *et al.* 2013; Wordofa 2014), suggested that oxidation potential can be promoted in higher pH conditions and higher persulfate dosage. However, some reports have shown that sulfate radical generation and their efficacy were not affected by pH (Lin *et al.* 2011; Xie *et al.* 2015). According to the results, a maximum disinfection performance was achieved in the combined UV/persulfate process in a pH range of 7 to 9. Tan *et al.* (Tan *et al.* 2013) attributed this effect to increased activation energy in the UV/persulfate system at a high pH and suggested two associated reasons. First, activated persulfate was augmented at alkaline pH (Equation (3)), and second, in the presence of hydroxyl ions (OH^-) some of the peroxydisulfate converts to hydroxyl radicals (Equation (4)) (Driedger *et al.* 2001). Generally, higher pH, contact time, and persulfate dosage result in a higher oxidation potential (Lin *et al.* 2011; Wordofa 2014; Xie *et al.* 2015).

$$S_2O_8^{2-} \xrightarrow{OH^-} 2SO_4^{\bullet -} \quad (3)$$

$$SO_4^{\bullet -} + OH^- \rightarrow SO_4^{2-} + OH^{\bullet} \quad (4)$$

Process optimization

Optimization formulas were obtained (Equations (5)–(7)) according to the regression models (Table 4) for UV and persulfate alone and the combined disinfection process.

$$Y_1 = 0.216537 + 0.056403\,X_1 - 0.080641\,X_2 \quad (5)$$

$$Y_2 = 0.047522 - 0.003002X_1 + 0.041435X_2 - 0.002442X_3 \quad (6)$$

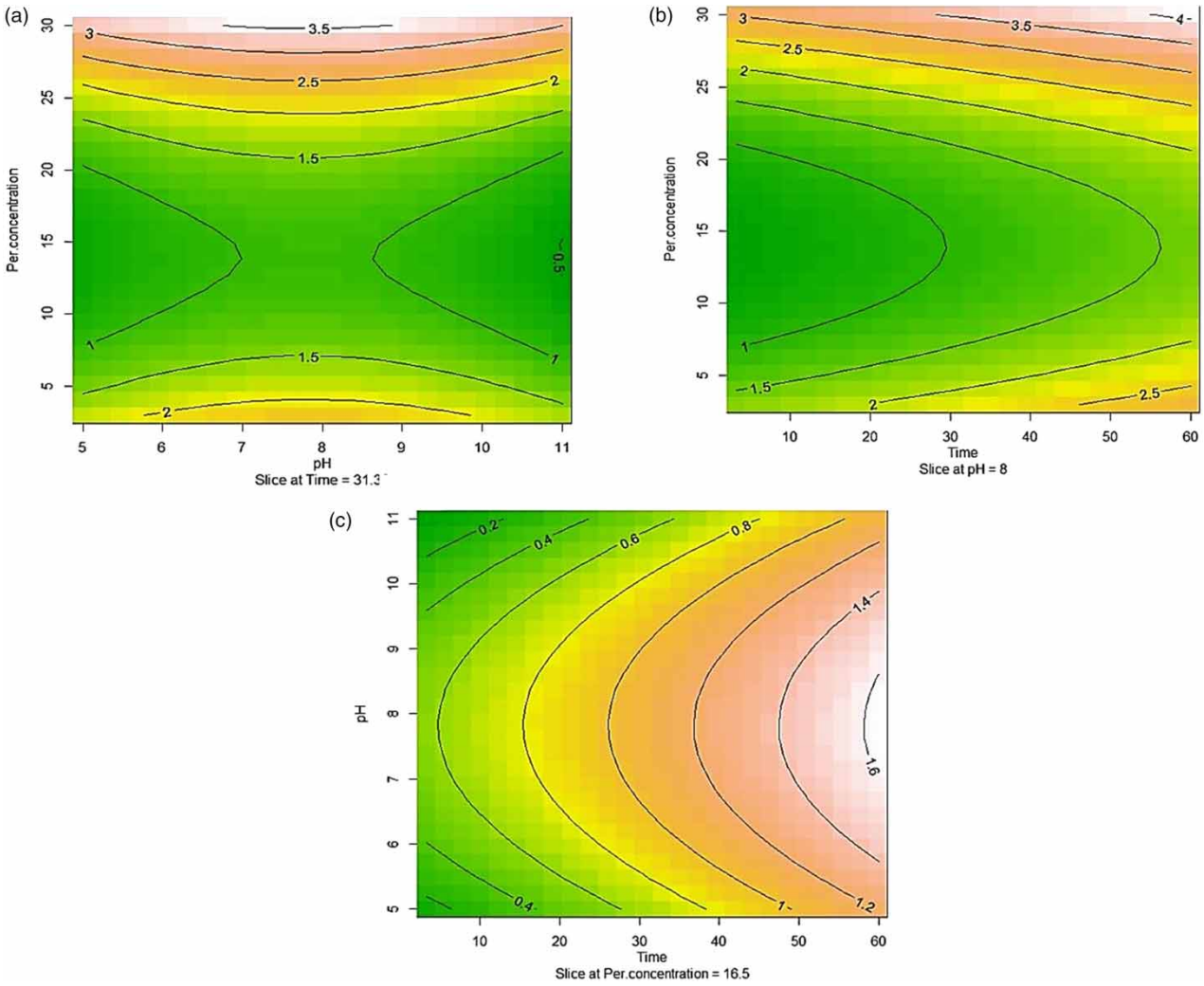

Figure 2 | Contour plot of log inactivation of *Bacillus subtilis* spores by a combined UV/persulfate process. Shows the effect of (a) persulfate concentration and pH, (b) persulfate concentration and contact time, (c) pH and contact time, and their mutual interactions on *B. subtilis* spore inactivation.

$$Y = -0.9398 + 0.0187X_1 + 0.8495\,X_2 - 0.2755\,X_3 - 0.0544\,X_2^2 + 0.00985\,X_3^2 \quad (7)$$

where Y_1, Y_2, Y_3 represent log inactivation of *B. subtilis* spores by the UV, persulfate and UV/persulfate processes, respectively; X_1 is the contact time; X_2 is the pH of the solution; and X_3 is the persulfate concentration in the sample. The above equations were solved using Solver add-Ins in Microsoft Excel 2013, and the optimization results for the three processes are presented in Table 6. Based on these results, the maximum inactivation of *B. subtilis* spores was predicted at 4.1 log after 60 min of treatment by the combined UV/persulfate process at pH 7.8 and 30 mM persulfate dosage. At this optimization point, the UV dose can be calculated by multiplying the lamp intensity

Table 6 | Optimization results of *B. subtilis* spore reduction by UV, persulfate and combined UV/persulfate processes

Process	Contact time (min)	pH	Persulfate dosage (mM)	Log inactivation
UV	60	5	–	3.19
Persulfate	5	11	3	0.48
UV/ Persulfate	60	7.8	30	4.1

(112 μW/cm^2) by optimum contact times (60 min), which is equal to 6.72 mW.s/cm^2. According to the results shown in Figures 1 and 2, and the results predicted in the optimized conditions, it is clear that the predicted amounts are consistent with those obtained in the laboratory. Although a higher amount of reduction out of the boundary of the space that was studied is possible, it does not seem to be practically feasible, since the conventional disinfection period is about 30–60 min. Therefore, the optimum reduction level was limited to values which are achievable by changing the independent variables as defined in the study.

Based on the optimal conditions, the disinfection potential of sulfate radicals in comparison with hydroxyl radicals generated by O_3/UV (Jung *et al.* 2008) and H_2O_2/UV (Cho *et al.* 2011) processes is 1.6 and 0.8 log higher in conditions similar to those used in this study, respectively. On the other hand, Wordofa (Wordofa 2014) reported 3.5 log inactivation of *E. coli* at lower persulfate concentrations, and longer contact times than those used in this research; however, it should be considered that spores are much hardier than bacteria.

Scanning electron microscopy images

Figure 3 shows scanning electron microscopy (SEM, ZEISS, DSM960A, Germany) images of the extent of spore coat damage of *B. subtilis* spores under optimized conditions conducted in the laboratory. As shown in Figure 3(a), the unexposed *B. subtilis* spores were ellipsoidal, and the main spore structure (i.e., inner and outer coat layers) was clear. Figure 3(b) shows the effects of UV irradiation on the spore's structure as pits in the spore's coat as well as transformed and amorphous morphology. A comparison of spores treated with persulfate (Figure 3(c)) and control spores (Figure 3(a)) revealed that the spore coats were unaffected, and no distinct differences were observed. Analysis

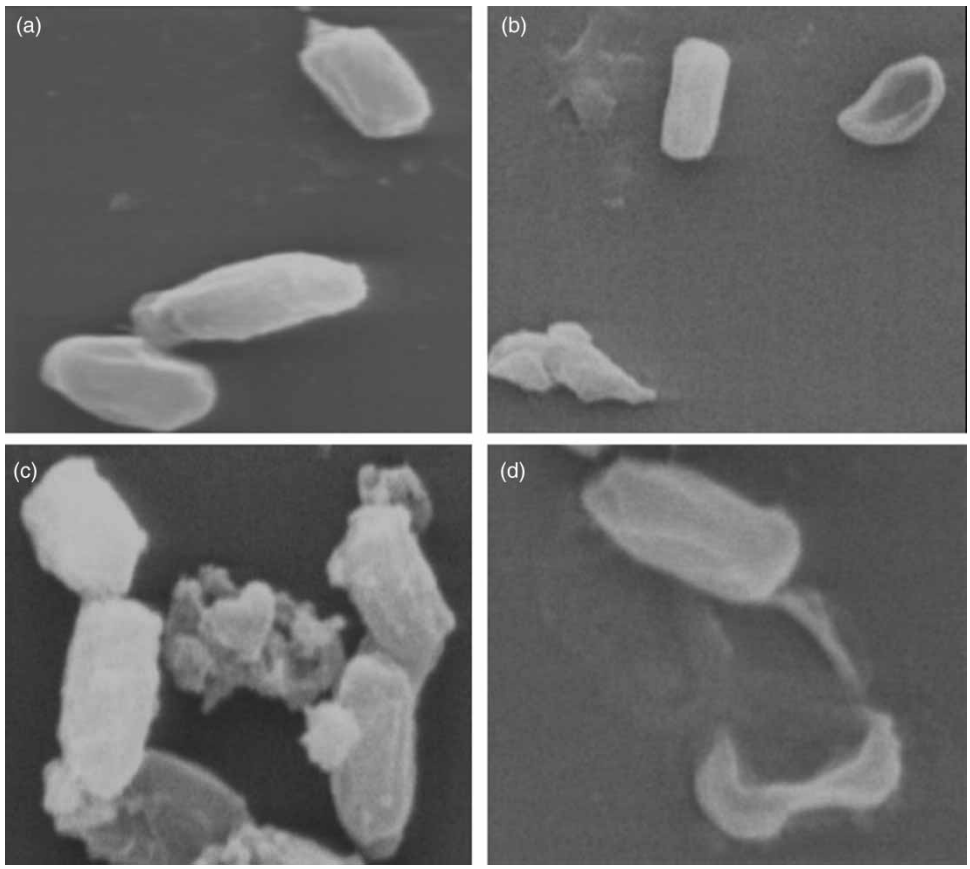

Figure 3 | Scanning electron microscope (SEM) images of *Bacillus subtilis* spores under optimal conditions in different processes. (a) Unexposed, (b) UV alone (t = 60 s, pH = 5), (c) persulfate (t = 5 s, pH = 11, C = 3 mM), (d) UV/persulfate (t = 60 s, pH = 7.8, C = 30 mM).

by SEM image showed damaged and ruptured *B. subtilis* spore coats and cores after treatment by combined UV/persulfate (Figure 3(d)).

Ultrastructural analysis of SEM images shows that *B. subtilis* spores were resistant to persulfate treatment but that UV irradiation was associated with damaged spore coat layers and that combined UV/persulfate could disrupt the spore coat and core with the highest efficiency. In fact, sulfate radicals were able to enter spores, and cellular compositions diffused into the outer perimeter of spores. This SEM image demonstrates a significant reduction in size and number and also amorphous morphologies of the spores after the combined UV/persulfate process.

In addition, Riesenman and Nicholson (Riesenman & Nicholson 2000) reported that the spore coat, particularly the inner coat layer, could not resist 254 nm UVC radiation. The destructive mechanisms underlying the disrupted spore coat and core were not the goal of this study and require further analysis.

CONCLUSIONS

Bacillus subtilis spores were used as target microorganisms that are resistant to conventional disinfectants. The effects of three disinfectants including UV, persulfate, and combined UV/persulfate were investigated for their efficacy in the inactivation of *B. subtilis* spores. In the case of each individual process, UV irradiation reduced spores up to 3.19 log in certain conditions (i.e., pH = 8 and 60 min contact time), while persulfate did not achieve a significant level of spore inactivation at higher applied dosages. Among the tested disinfectants, the combined UV/persulfate process demonstrated the greatest effect on spore inactivation with a 4.1 log reduction. In this process, $SO_4^{\bullet-}$ radicals generated from the persulfate activation by UV irradiation showed an enhancement of inactivated *B. subtilis* spores with close to a 1 log reduction. Although the results achieved in the UV/persulfate process are promising for spore inactivation, additional studies are required to evaluate other aspects such as the various UV intensity, process cost, and bench scale use of this process.

Also it should be noted that the current study was performed to evaluate the efficacy of the process; so we had to test higher spore densities to see the effects of independent variables on the log-reduction of spores. Then, the applied amount of persulfate could tend to high values of sulfate at the end of disinfection periods. Obviously, in real conditions we are not faced with such high levels of spores; so lower amounts of persulfates would be sufficient. Therefore, further investigation is required to include initial spore concentrations and lower persulfate levels as independent variables.

ACKNOWLEDGEMENTS

This study was supported by Tehran University of Medical Sciences under research fund grant No. 94-01-27-28523 to Dr Ramin Nabizadeh.

REFERENCES

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S. & Escaleira, L. A. 2008 Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* **76** (5), 965–977.

Blatchley III, E. R., Meeusen, A., Aronson, A. I. & Brewster, L. 2005 Inactivation of *Bacillus* spores by ultraviolet or gamma radiation. *Journal of Environmental Engineering* **131** (9), 1245–1252.

Carley, K. M., Kamneva, N. Y. & Reminga, J. 2004 Response surface methodology, DTIC Document.

Cho, M., Kim, J.-H. & Yoon, J. 2006 Investigating synergism during sequential inactivation of *Bacillus subtilis* spores with several disinfectants. *Water Research* **40** (15), 2911–2920.

Cho, M., Gandhi, V., Hwang, T.-M., Lee, S. & Kim, J.-H. 2011 Investigating synergism during sequential inactivation of MS-2 phage and *Bacillus subtilis* spores with UV/H_2O_2 followed by free chlorine. *Water Research* **45** (3), 1063–1070.

Driedger, A., Staub, E., Pinkernell, U., Mariñas, B., Köster, W. & von Gunten, U. 2001 Inactivation of *Bacillus subtilis* spores and formation of bromate during ozonation. *Water Research* **35** (12), 2950–2960.

Facile, N., Barbeau, B., Prévost, M. & Koudjonou, B. 2000 Evaluating bacterial aerobic spores as a surrogate for *Giardia* and *Cryptosporidium* inactivation by ozone. *Water Research* **34** (12), 3238–3246.

Gao, Y.-Q., Gao, N.-Y., Deng, Y., Yang, Y.-Q. & Ma, Y. 2012 Ultraviolet (UV) light-activated persulfate oxidation of sulfamethazine in water. *Chemical Engineering Journal* **195**, 248–253.

Huang, X., Lu, Z., Bie, X., Lü, F., Zhao, H. & Yang, S. 2007 Optimization of inactivation of endospores of *Bacillus cereus* by antimicrobial lipopeptides from *Bacillus subtilis* fmbj strains using a response surface method. *Applied Microbiology and Biotechnology* **74** (2), 454–461.

Jung, Y. J., Oh, B. S. & Kang, J.-W. 2008 Synergistic effect of sequential or combined use of ozone and UV radiation for the disinfection of *Bacillus subtilis* spores. *Water Research* **42** (6), 1613–1621.

Jung, H., Kim, D., Gweon, B., Moon, S. & Choe, W. 2010 Enhanced inactivation of bacterial spores by atmospheric pressure plasma with catalyst TiO_2. *Applied Catalysis B: Environmental* **93** (3), 212–216.

Khuri, A. I. & Mukhopadhyay, S. 2010 Response surface methodology. *Wiley Interdisciplinary Reviews: Computational Statistics* **2** (2), 128–149.

Larson, M. A. & Mariñas, B. J. 2003 Inactivation of *Bacillus subtilis* spores with ozone and monochloramine. *Water Research* **37** (4), 833–844.

Lenth, R. V. 2009 Response-surface methods in R, using rsm. *Journal of Statistical Software* **32** (7), 1–17.

Lin, Y.-T., Liang, C. & Chen, J.-H. 2011 Feasibility study of ultraviolet activated persulfate oxidation of phenol. *Chemosphere* **82** (8), 1168–1172.

Lutze, H. V., Kerlin, N. & Schmidt, T. C. 2015 Sulfate radical-based water treatment in presence of chloride: formation of chlorate, inter-conversion of sulfate radicals into hydroxyl radicals and influence of bicarbonate. *Water Research* **72**, 349–360.

Mamane, H., Shemer, H. & Linden, K. G. 2007 Inactivation of *E. coli*, *B. subtilis* spores, and MS2, T4, and T7 phage using UV/H_2O_2 advanced oxidation. *Journal of Hazardous Materials* **146** (3), 479–486.

Mezule, L., Reimanis, M., Krumplevska, V., Ozolins, J. & Juhna, T. 2014 Comparing electrochemical disinfection with chlorination for inactivation of bacterial spores in drinking water. *Water Science & Technology: Water Supply* **14** (1), 158–164.

Post, G. B., Atherholt, T. B. & Cohn, P. D. 2011 Health and aesthetic aspects of drinking water. In: *Water Quality and Treatment: A Handbook on Drinking Water*, 6th edn (J. K. Edzwald, ed.). American Water Works Association. McGraw-Hill, New York.

Radziminski, C., Ballantyne, L., Hodson, J., Creason, R., Andrews, R. C. & Chauret, C. 2002 Disinfection of *Bacillus subtilis* spores with chlorine dioxide: a bench-scale and pilot-scale study. *Water Research* **36** (6), 1629–1639.

Riesenman, P. J. & Nicholson, W. L. 2000 Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. *Applied and Environmental Microbiology* **66** (2), 620–626.

Roshani, B. & Leitner, N. K. V. 2011 Effect of persulfate on the oxidation of benzotriazole and humic acid by e-beam irradiation. *Journal of Hazardous Materials* **190** (1), 403–408.

Tan, C., Gao, N., Deng, Y., Zhang, Y., Sui, M., Deng, J. & Zhou, S. 2013 Degradation of antipyrine by UV, UV/H_2O_2 and UV/PS. *Journal of Hazardous Materials* **260**, 1008–1016.

Wordofa, D. N. 2014 *Application of Iron Activated Persulfate for Disinfection in Water Treatment*. MSc Thesis, University of California.

Xie, P., Ma, J., Liu, W., Zou, J., Yue, S., Li, X., Wiesner, M. R. & Fang, J. 2015 Removal of 2-MIB and geosmin using UV/persulfate: contributions of hydroxyl and sulfate radicals. *Water Research* **69**, 223–233.

First received 1 June 2016; accepted in revised form 10 August 2016. Available online 20 August 2016