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A comparative study of the disinfection efficacy of H_2O_2/ferrate and UV/H_2O_2/ferrate processes on inactivation of *Bacillus subtilis spores* by response surface methodology for modeling and optimization

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ABSTRACT

Although chlorination can inactivate most of the microorganisms in water but protozoan parasites like C. parvum oocysts and Giardia cysts can resist against it. Therefore, many researches have been conducted to find a novel method for water disinfection. Present study evaluated the synergistic effect of H2O2 and ferrate followed by UV radiation to inactivate Bacillus subtilis spores as surrogate microorganisms. Response surface methodology(RSM) was employed for the optimization for UV/H2O2/ferrate and H2O2/ferrate processes. By using central composite design(CCD), the effect of three main parameters including time, hydrogen peroxide, and ferrate concentrations was examined on process performance. The results showed that the combination of UV, H2O2 and ferrate was the most effective disinfection process in compare with when H2O2 and ferrate were used. This study indicated that by UV/H2O2/ferrate, about 5.2 log reductions of B. subtilis spores was inactivated at 9299 mg/l of H2O2 and 0.4 mg/l of ferrate concentrations after 57 min of contact time which was the optimum condition, but H2O2/ferrate can inactivate B. subtilis spores about 4.7 logs compare to the other process. Therefore, the results of this research demonstrated that UV/H2O2 /ferrate process is a promising process for spore inactivation and water disinfection.

1. Introduction

Giardiasis is a global disease and *Giardia lamblia* cyst can survive for a long time even at low temperature in water. Cryptosporidiosis outbreaks have been reported since 1983 in America (Craun et al., 2010). More prevalence of Giardiasis and Cryptosporidiosis has been observed in industrialized countries. The main symptoms of these protozoa are mild to severe diarrhea and possibly shorten the life of those who are at the risk of immune deficiency such as AIDS. About 1.5 million people are estimated to die every year from diarrhea as the result of inadequate sanitation and drinking unsafe water. Based on the data, the major reason for this type of disease is water contamination with sewage and low efficiency of water treatment systems. According to the latest reports, 748 million people did not access to drinking water in 2012 (Tsydenova et al., 2015). According to the above description, there is a vital need to remove and inactivate these pathogens and their related diseases. Therefore disinfection process is one of the most important steps in water and wastewater treatment facilities (Yousefzadeh et al., 2014).

Nowadays, among many disinfectants used for water and wastewater disinfection, chlorination is more common. But although chlorination can inactivate most of the microorganisms in water such as *Escherichia coli*, but protozoan parasites such as *Cryptosporidium parvum* oocysts and *Giardia* cysts can resist against it. On the other hand, scientists concern about the cancer potential of chlorine by producing disinfection by-products (DBPs) during drinking water treatment (Chu et al., 2013). Therefore, in recent years, many studies have been conducted to find novel methods for water disinfection. Consequently, it is

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critical to evaluate the performance of innovative and effective methods of disinfection in order to replace current methods (Yousefzadeh et al., 2014).

Nowadays, the application of hybrid (combined) or synergistic techniques has become prevalent all over the world; this approach which seems to be the best alternative for water disinfection (Cho et al., 2006a). Advanced oxidation processes (AOPs) are one of these methods. Among different conventional processes, UV-based advanced oxidation processes could be used in various applications such as decomposition of hazardous organic compounds and natural organic matters (NOMs) as well as treating compounds that produce taste and odor in water (Cho et al., 2011). These processes include O_3/H_2O_2, O_3/UV, UV/H_2O_2, TiO_2/UV, H_2O_2/catalyst/Fenton and photo-Fenton processes. Photochemical and photo-catalytic AOPs are based on UV radiation as an external source of energy (Schulte et al., 1995; Zhang et al., 2014).

Absorption of UV light by H_2O_2 results in the photolysis of the peroxidic bond, which consequently leads to produce highly reactive radicals (Eq. (1)). Hydroxyl radical (OH) is a strong disinfectant which destroys hazardous contaminants in water and is responsible for the inactivation of microorganisms. This process acts with two main mechanisms for disinfecting purposes: 1. Oxidation and destruction of wall and membrane of microorganisms, 2. Enzyme inactivation due to the diffusion of disinfectant inside the cell (Cho et al., 2011; Mamane et al., 2007; Unosson et al., 2013).

$$H_2O_2 + hv \rightarrow 2OH \quad (1)$$

Furthermore, ferrate (Fe (VI), +6 oxidation state of iron) is another powerful oxidant that has the redox potentials of −2.2 and 0.72 V in acid and base media, respectively (Eqs. (2) and (3)). Ferrate is a green synthesis material and acts as a multi–purpose chemical (oxidant, coagulant, disinfectant) for water treatment (Jiang et al., 2006; Sharma et al., 2005).

$$FeO_4^{2-} + 8H^+ + 3e^- \rightarrow Fe^{3+} + 4H_2O \qquad E^\circ = +2.20V \quad (2)$$

$$FeO_4^{2-} + 4H_2O + 3e^- \rightarrow Fe(OH)_3 + 5OH^- \quad E^\circ = 0.72V \quad (3)$$

This technology is able to remove unconventional and emerging microorganisms and can eliminate different contaminants; it not only eliminates toxins, but also generates non–hazardous products and non–toxic DBPs (Jiang et al., 2006; Sharma et al., 2005). Moreover, potassium ferrate (K_2FeO_4) can be used in a wide range of pH and many studies have reported that ferrate is stable in alkaline conditions and its decomposition rate is slow (Cho et al., 2006b; Jiang et al., 2007).

Therefore the aim of the present study was to evaluate the synergistic effect of hydrogen peroxide and ferrate when coupled with UV radiation on the disinfection and inactivation of chlorination resistance microorganism such as *Cryptosporidium parvum* oocysts and *Giardia* cysts. However, due to their analysis limitations, there is no possibility for the direct measurement of the mentioned pathogens. Moreover, as these organisms are pathogenic, their study in laboratory conditions is difficult, expensive, and time-consuming; consequently, they are not suitable for routine monitoring. Therefore, *B. subtilis* spore has been chosen as a surrogate indicator for mentioned pathogens, which is easy to count and identify in laboratory conditions (Hamer, 2011). *B. subtilis* is a Gram-positive, aerobic, and non-pathogenic organism that is naturally found in soil and vegetation. In addition, this microbe can form spore when the nutrients in the environment are limited. Moreover, spore of *B. subtilis* is highly resistant to disinfection (Leggett et al., 2015).

So according to the above mentioned, the objective of this study was to compare the potential inactivation effects of hydrogen peroxide and ferrate and the simultaneous process of UV/H_2O_2/ferrate on the disinfection and inactivation of *B. subtilis* spore. In the present study, the central composite design (CCD) used for designing the steps of study along with response surface methodology (RSM) was used for modeling and optimization of the reduction log of *B. subtilis* spore different disinfection process.

2. Material and methods

2.1. Experimental design

A central composite design and response surface methodology were applied to investigate the optimum conditions for the inactivation of *B. subtilis* spores. A central composite design is a second-order factorial design which includes a central point and axial points. With this design, the number of tests can be reduced and all the coefficients of quadratic regression model and interaction factors can be estimated. If the number of independent variables in this design is considered k, the points of factorial experiments are 2^k and axial points are 2k. Therefore, if the number of repetitions at a central point is n, the total number of experiments is $2^k + 2k + n$ (Lenth, 2009; Montgomery, 2012).

For the experimental design, the first step is to select the independent variables and delimit the experimental region (Azari et al., 2015). In the present study, contact time (X_1, 5–60 min), H_2O_2 concentration (X_2, 0–10000 mg/l), and ferrate concentration (X_3, 0–15 mg/l) were selected as the independent variables (with the five levels shown in Table 1) and log inactivation (Y) as the dependent variable were examined with 29 experiments (Table 2). The ranges of the variables were selected according to the performed pre-tests and screening previous studies. The code and level of the factors are shown in Table 1. The variables were coded according to Eq. (4):

$$\mathscr{X}_i = (X_i - X_0)/\Delta X \quad (4)$$

where $X_i$is the actual value of variable, X_0 is the value of $X_i$at the central point, and ΔX is the step change.

This design includes three independent variables, at five levels, two blocks, and 15 repetitions at the central point (to calculate repeatability of process) that is used in order to investigate the impact of disinfection and optimization process. The experiments were conducted in two phases including combined H_2O_2/ferrate in the presence and absence of UV.

2.2. Preparing microbial culture (spore production)

As described before, *B. subtilis* spores were selected as the target in this work. For this purpose, a freeze–dried lyophilized strain of *B. subtilis* (ATCC 6638- in vegetative form) was obtained from Pasteur Institute of Iran. In the first step, *B. subtilis* was initially cultivated on tryptic soy agar (TSA, Merck) for 24 h at 35 °C. Then, in order to induce sporulation, the harvesting colony from TSA was cultivated on antibiotic assay medium1 (Himedia, M003) containing 0.3 g/l MnO_4 and providing an extended incubation period (5–7 days) at 37 °C. After incubation, the bacterial inocula were prepared by washing and suspending the colonies in 0.9% physiological saline solution and transferred to 10 ml sterile centrifuge tubes. Finally, for purification, the suspensions were centrifuged (5000 rpm, 10 min) and kept in the refrigerator at 4 °C (Radziminski et al., 2002; Sabeti et al., 2016).

Table 1
Independent variables and their values.

Independent variables	Symbol	Code and relevant level [a]				
		$-\alpha\sqrt{2}$	−1	0	1	$+\alpha\sqrt{2}$
Contact Time (min)	X_1	5	16.1	32.5	48.8	60
H_2O_2 Concentration (mg/l)	X_2	0	2026.9	5000	7973.0	10000
Ferrate Concentration (mg/l)	X_3	0	3	7.5	11.9	15

[a] $X_i = (\text{Time}-32.5)/27.5$.$X_i = (H_2O_2\text{Con} - 5000)/5000 X_i = (\text{FerrateCon}-7.5)/7.5$

Table 2
Arrangement of CCD for the three independent variables used in both study phases and obtained results.

Run NO.	Time (min)	H_2O_2 Concentration (mg/l)	Ferrate Concentration (mg/l)	Log inactivation (Y)	
				H_2O_2/ Ferrate	UV/ H_2O_2/ Ferrate
1	32.5	5000	7.5	1.12	2.18
2	32.5	5000	7.5	0.82	2.60
3	16	2027	12	0.30	1.30
4	32.5	5000	7.5	1.24	2.37
5	32.5	5000	7.5	1.20	1.90
6	49	7973	3	3.30	4.44
7	32.5	5000	7.5	1.18	2.40
8	16	7973	12	1.00	2.00
9	49	2027	12	1.60	2.65
10	32.5	5000	7.5	1.04	2.14
11	32.5	5000	7.5	1.89	2.36
12	49	2027	3	0.70	3.40
13	16	2027	3	0.03	1.64
14	32.5	5000	7.5	1.30	2.90
15	49	7973	12	2.02	4.05
16	16	7973	3	0.90	2.90
17	32.5	5000	7.5	0.77	2.15
18	32.5	5000	7.5	0.92	2.24
19	32.5	10000	7.5	2.75	4.27
20	32.5	5000	0	0.70	1.67
21	32.5	5000	7.5	0.34	2.11
22	32.5	5000	15	0.65	2.52
23	5	5000	7.5	0.40	1.00
24	32.5	5000	7.5	0.85	2.67
25	32.5	0	7.5	0.10	1.59
26	60	5000	7.5	2.18	3.18
27	32.5	5000	7.5	1.04	2.56
28	32.5	5000	7.5	0.59	1.74
29	32.5	5000	7.5	0.76	2.60

2.3. Sample preparation

Tap water was used for preparing the aqueous solutions. Also, 4 ml of spore suspension was poured into 500 ml of sterile tap water for sample preparation. Heat shock (80 °C for 10–12 min) was used to ensure that all the remaining vegetative cells were removed. The inoculate was enumerated and adjusted by plate count method after dilution. According to this method of preparation, the initial loading rates of 10^7 to 10^8 spores per ml were prepared.

2.4. Experimental procedure

The experiments were designed to examine the inactivation of *B. subtilis* spore resulted from H_2O_2/ferrate or UV/H_2O_2/ferrate processes. The testing process step-by-step is as follow:

2.4.1. Disinfection procedure

Initially, 50 ml of bacterial sample, prepared in the previous step (step 2.3), was poured into a sterile Petri dish with 90 mm of diameter and open surface to the atmosphere. Then, for the first study step (H_2O_2/ferrate experiments), different concentrations of H_2O_2 (directly, 0–10 g/l for H_2O_2 15%w/w) and ferrate (0–15 mg/l from stock solution) according to the experimental design concentration for each run (Table 2) were added to the Petri dish and the samples were gently stirred during different contact times. The second study step (UV/H_2O_2/ferrate experiments) was designed similar to the first, but in the presence of UV radiation. In this study, the UV intensity was constant and at the wavelength of 254 nm. When the distance between the UV lamp and water was equal to 125 mm, it was 1.12 w/m^2.

2.4.2. Disinfectant neutralization

At the end of each contact time, 4.0% w/v of sodium thiosulfate and less than 0.2 mg/l catalase solution as the auxiliary neutralizer agent were added to quench and neutralize the residual disinfectant. Both of these neutralizers are not toxic to the *B. subtilis* spores.

2.4.3. Microbiological analysis

After neutralization process, 10 ml of the disinfected sample was collected for microbial analysis. Microbiological analyses of the samples were performed before and after the disinfection process. Viability of the spores was analyzed by membrane filtration technique as described in standard methods (method 9610 D) (APHA et al., 2005). For this purpose, the blank samples and neutralized mixtures were repeatedly diluted by transferring 10 ml of the solution to 90 ml of sterile 0.9% saline serum (ten-fold serial dilutions). Then, the number of viable spores was determined by spreading and filtering 1 mL of the mixture on the surface of nitrocellulose filter (sartorius stedim, No.11406) with diameter of 47 mm and 0.45 μm of nominal pore size.

After filtration, the filters were placed in a Petri dish containing nutrient agar (Merck, No 1.05450) and 0.015 mg/l of trypan blue (Titrachem, No.512). Bacterial plates were incubated for 24 h at 37 °C and, then, the number of colonies was counted (Larson and Mariñas, 2003). Finally, the log inactivation of spores or Y as response or dependent variable can be was calculated by Eq. (5):

$$Y = \log_{10} N_0/N_t \quad (5)$$

where N_0 and N_t are the initial number of spores (CFU/ml) and final number of survivals (CFU/ml), respectively.

2.4.4. Analysis the effect of pH

At the final stage, the inactivation kinetics of *B. subtilis* spores with both disinfection processes was examined to determine the effect of pH. It should be noted that according to the standards, the final pH level of drinking or treated water should be close to 7 as alkaline and acidic pH has many adverse effects on health and environment. In addition adjusting pH to higher and lower levels needs addition of chemicals and consequently can increase the treatment costs which are not also feasible in real and large scale applications. So, in the present study the impact of pH was not evaluated directly in the CCD design and it was investigated separately. For this aim, one study run (center point: contact time = 32.5 min, H_2O_2 concentration = 5000 mg/l, and ferrate concentration = 7.5 mg/l) was selected and the effect of pH within the range of 5–11 was investigated. pH was adjusted to the desired value by a few drops of HCl or NaOH (0.5 N). It should be highlighted that the initial pH of the solution without adjustment was 8.

2.5. Data analysis

As before mentioned for this study the experiments were conducted by Central Composite Design (CCD) and the experimental data were analyzed by statistical package of R software (version 3.1.3). Multiple regression analysis for both phases is shown in Table 3. Multiple R^2, adjusted R^2, and lack of fit tests were used to assess the model adequacy. P–value was used as a tool to check the significance of coefficients. P–value of less than 0.05 indicates that the model is significant (Hasan et al., 2011; Sabeti et al., 2016).

3. Results and discussion

As previously mentioned, the CCD design for different variables (contact time and concentration of H_2O_2 as well as ferrate) and the value of inactivation log for spores of *B. subtilis* (Y) obtained under different experimental conditions for H_2O_2/ferrate and UV/H_2O_2/ferrate processes (the first and second study phases) are summarized in Table 2.

Table 3
Regression coefficients and their significance in the quadratic model.

Model term	Coefficient estimate	Standard error	T value	Pr (> |t|)
H_2O_2/Ferrate				
Intercept	0.95	0.090	10.75	3.16e-10 ***
X_1 – Time	1.03	0.17	6.07	4.13e-06 ***
X_2 – H_2O_2 Concentration	1.12	0.17	6.55	1.35e-06 ***
X_3 – Ferrate Concentration	−0.01	0.17	−0.07	0.94
X_2:X_3	−0.83	0.37	−2.22	0.04 *
X_1^2	0.32	0.25	1.25	0.22
X_2^2	0.45	0.25	1.78	0.088
R^2: 0.80, Adjusted R^2: 0.78, F-statistic:15 on 6 and 22 DF, p-value: 8.79e-07				
UV/H_2O_2/Ferrate				
Intercept	2.32	0.09	25.26	< 2.2e-16 ***
X_1 – Time	1.27	0.17	7.31	1.94e-07 ***
X_2 – H_2O_2 Concentration	1.10	0.17	6.30	1.96e-06 ***
X_3 – Ferrate Concentration	−0.116	0.17	−0.67	0.51
X_1^2	0.006	0.26	0.026	0.98
X_2^2	0.85	0.26	3.26	0.003 **
R^2: 0.82, Adjusted R^2: 0.75, F-statistic: 20.88 on 5 and 23 DF, p-value: 7.34e-08				

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

3.1. Effect of H_2O_2/ferrate

Table 3 Presents a regression coefficients and their significance in the quadratic model and in the other hand Table 4 presents a summary of analysis of variance (ANOVA) for the selected quadratic model for both study phases.

As shown in Table 3, the results of the reduced quadratic model for H_2O_2/ferrate process (first study phase) can demonstrate that all the linear coefficients, except ferrate concentration and quadratic coefficient, including H_2O_2 concentration (X_2^2) and interaction between H_2O_2 and ferrate concentration (X_2: X_3) were statistically significant ($P < 0.05$). According to the closeness of coefficient of determination values of 0.80 and 0.78 for R^2 and R^2adj, respectively, and the lack of fit of 0.36, it can be stated that the obtained experimental data can be fitted with the model with a high degree of precision and, consequently, this model is acceptable (Tables 3 and 4).

Also the response can be represented graphically, either in the three dimensional space or as a contour plot that help visualize the shape of the response surface analysis. Initially, the effect of H_2O_2/ferrate process (first study phase) on the inactivation of *B. subtilis* spores was investigated.

The *B. subtilis* spore reduction as a function of H_2O_2 concentration and time at the constant concentration of 7.5 mg/l of ferrate is shown in Fig. 1a. As this figure shown, with increasing time (from 5 to 60 min) and H_2O_2 concentration, the greater spore reduction of *B. subtilis* (about 4 log inactivation) was achieved.

Fig. 1b presents the effect of time and ferrate concentration at constant H_2O_2 concentration equal to 5000 mg/l. It can be observed that as time increased, the reduction of *B. subtilis* spores increased, but in this condition, the concentration of ferrate had an insignificant effect. Fig. 1c represents the response surface plot for the interaction of H_2O_2 and ferrate concentration at the constant time of 32.5 min. According to this figure, increasing H_2O_2 concentration from 5000 to 10000 mg/l and gradually increasing ferrate concentration led to improving the efficiency of *B. subtilis* reduction from 1 to 3 log.

As mentioned before, ferrate has high potential as a multipurpose water treatment. Cho et al. in their study stated that the inactivation efficiencies of Fe(VI) is a suitable method for *E.coli* (Cho et al., 2006b). In another study, Jiang and Wang reported that the potassium ferrate (VI) is a highly efficient for *E.coli* inactivation in compare with sodium hypochlorite. In order to achieve the same *E.coli* inactivating with ferrate, less dose and contact time were required. Also in this situation (less affected by solution pH) the disinfection rate of the ferrate was faster (Jiang et al., 2007). However the study of Gombos et al. showed that less than 8 mgL^{-1} Fe(VI) dose with 30 min contact time was sufficient for 99.9 bacterial inactivation in municipal secondary effluent (Gombos et al., 2012).

In most investigated study, ferrate is used for inactivation of bacteria in waste water especially *E. coli* and in our study we focus on resistant bacteria like *B. subtilis*.

It is interesting to note that other disinfectants have been previously used by other researchers for inactivating *B. subtilis* spore. Foegeding (1985) and Young and Setlow (2003) reported that the spore coat as the primary protective barrier against ozone inactivation as the *bacillus* and *clostridium* species were rapidly inactivated when their spore coat were removed (Foegeding, 1985; Young and Setlow, 2003). In similar experiments, Young and Stelow reported that lacking of spore coat of *B. subtilis* resulted in its rapid inactivation by chlorine and chlorine dioxide.

In the studies mentioned above, the bacteria were less resistant, but in our study with high bacteria resistance, the inactivation was higher than them. Therefore, we cannot exactly compare the results of this research with other studies, but what is clear is that the disinfection efficacy of H_2O_2/ferrate against *B. subtilis* spores is more than other processe

Table 4
Analysis of variance (ANOVA) for regression equation (reduced models).

Source	DF	Sum of squares	Mean square	F value	Prob > F
H_2O_2/Ferrate					
Model	2	0.72	0.36	2.61	0.096
Residuals	22	3.07	0.14	–	–
Lack of fit	8	1.25	0.16	1.21	0.36
Pure error	14	1.81	0.13	–	–
UV/H_2O_2/Ferrate					
Model	2	1.58	0.79	5.38	0.01
Residuals	23	3.38	0.15	–	–
Lack of fit	9	2.04	0.23	2.38	0.07
Pure error	14	1.33	0.09	–	–

3.2. Effect of UV/H_2O_2/ferrate

The results of UV/H_2O_2/ferrate process (second study phase-second part of Tables 3 and 4) indicated that the linear variables including contact time (X_1) and H_2O_2 concentration (X_2) as well as the quadratic variable including H_2O_2 concentration (X_2^2) were significant while ferrate concentration (X_3) was not significant. No synergistic effect was observed when ferrate was applied after UV/H_2O_2. Moreover, as no interactions were observed between the variables, and their interactions were not statistically significant, they were eliminated from the final model and the reduced models were presented for optimization. Insignificant difference between R^2 (0.82) and R^2 adj (0.75), which was less than 0.2, can indicate that this reduced model fits with experimental data with a high degree of precision. It is interesting to note that in multiple regressions analysis, the adjusted R squared gives an idea of how the model is generalized. In an ideal situation such as obtained results of the present study, it is preferable that its value be as close as possible to the value of R squared. Therefore, the proportion of the variance explained by the multiple regression models is indicated by R squared. In addition, according to the obtained p–value (7.34×10^{-8}), the reduced model's adequacy fit was obvious. Furthermore, the lack of fit of this model was 0.07 which was more than 0.05 and indicated that the model fitted the data with a high degree of precision (Table 4).

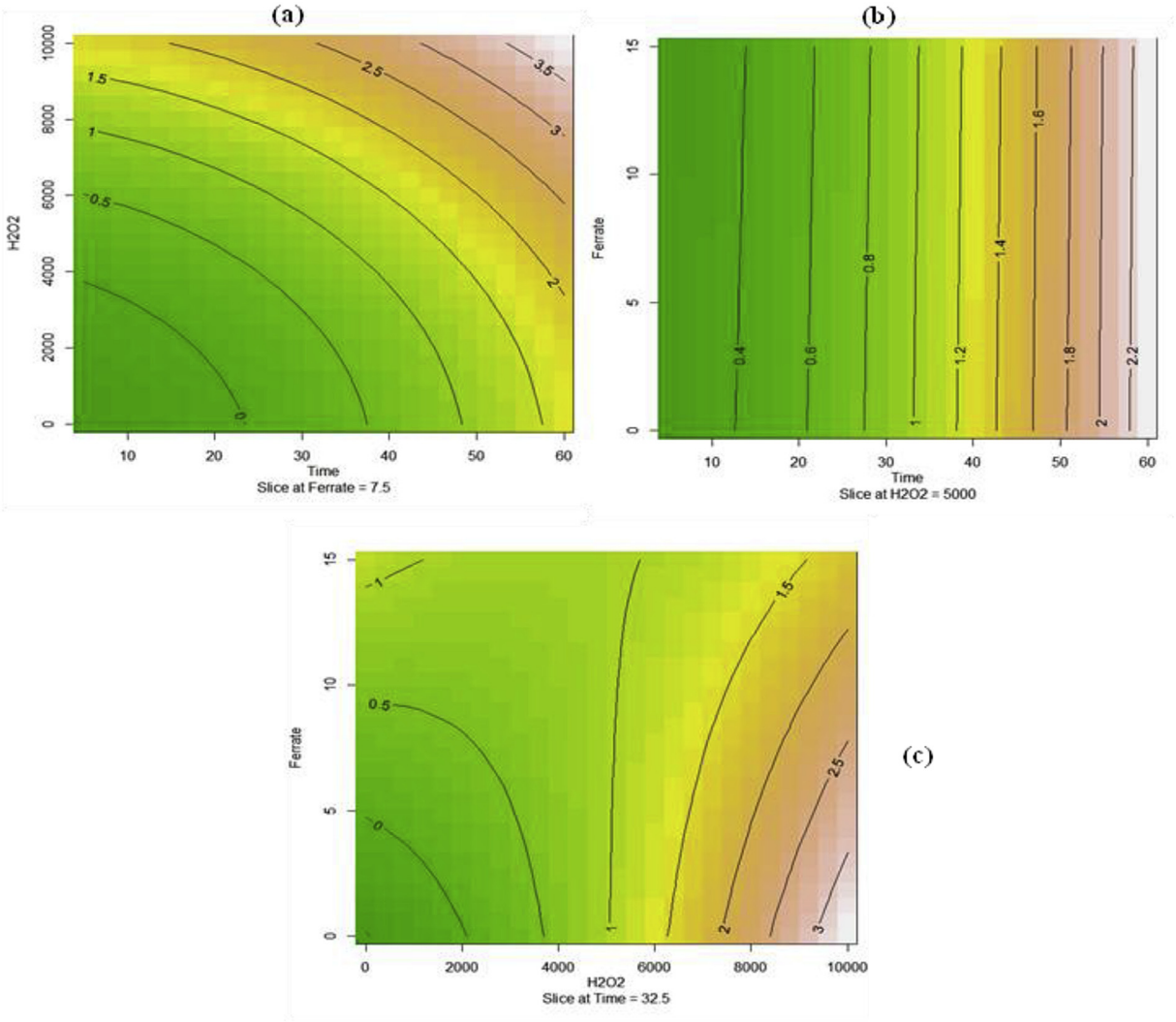

Fig. 1. Response surface plot and its corresponding contour plot of H_2O_2/ferrate process (a) effect of time and H_2O_2 concentration, (b) effect of time and ferrate concentration, (c) effect of H_2O_2 and ferrate concentrations, and their log inactivation on *B. subtilis* spores.

Also the effect of H_2O_2 and ferrate process (with UV- second phase of study) on *B. subtilis* spores inactivation is shown on Fig. 2. Fig. 2 a presents the effect of time and H_2O_2 variations at the constant concentration of 7.5 mg/l of ferrate. From Fig. 2 a, it can be observed that the log inactivation of *B. subtilis* spores increased up to 5 log of reduction as the contact time and H_2O_2 concentration increased. The contour plots in Fig. 2b and c demonstrate that in the applied range of ferrate concentrations as an independent variable, there was no effect on the inactivation of *B. subtilis* spores; only by prolonging and extending the contact time (from 0 to 60 min) and increasing the concentration of H_2O_2 (from 0 to 10000 mg/l), the inactivation efficiency of *B. subtilis* spores increased.

Analysis of variance (ANOVA) indicated that the important factors for the inactivation of *B. subtilis* were contact time and addition of hydrogen peroxide, and the results were in good agreement with the previously published data. As mentioned before, in combination of UV and H_2O_2 (phase 2), UV radiation can hit the spores and therefore can penetrate inside the spores so under these circumstances H_2O_2 can be more effective (Cho et al., 2006a; Mamane et al., 2007). According to the results by Mamane et al. (2007), *B. subtilis* spores did not inactivate in any of the treatment processes (i.e. H_2O_2, UV > 295 nm, and UV/H_2O_2) and it was stated that the spores exposed to UV radiation at longer wavelengths exhibited different DNA photochemistry. Actually they did not obtain any spore inactivation at higher wavelength. (Mamane et al., 2007). The results of their study indicated that hydrogen peroxide alone cannot affect spores because spore coat serves as a barrier for the diffusion of H_2O_2, while OH radicals by UV radiation had the highest efficiency in the reduction of the microorganism. According to Cho et al. 2 log reduction of *B. subtilis* spore was achieved at both pH 5.6 and 8.2 (Cho et al., 2006a). In addition, Cho et al. (2011) believed that addition of H_2O_2 was more effective in enhancing inactivation of *B. subtilis* spores (Cho et al., 2011).

Generally, UV radiation and hydrogen peroxide can produce highly reactive hydroxyl radicals (Crittenden et al., 2012). According to the analysis of figures, addition of hydrogen peroxide and appropriate contact time were two critical variables for the inactivation of *B. subtilis* spores. Oxidation rate can be increased by increasing the concentration of H_2O_2 and extending time, which leads to more hydroxyl radical production as the adsorption of UV rays by H_2O_2 increases (Shu et al., 2013). According to some studies, excessive concentrations of H_2O_2 have a slight effect on process performance or even reduce the oxidation rate (Rezaee et al., 2014). Probably high concentrations of hydrogen peroxide act as a radical scavenging agent and reduce the amount of active radicals.

It should be mentioned that effect of the combination of UV light, H_2O_2 and ferrate on inactivation of *B. subtilis* spores has been not previously studied. Therefore, we cannot exactly compare the results of this research with other studies but there are some studies that focus on

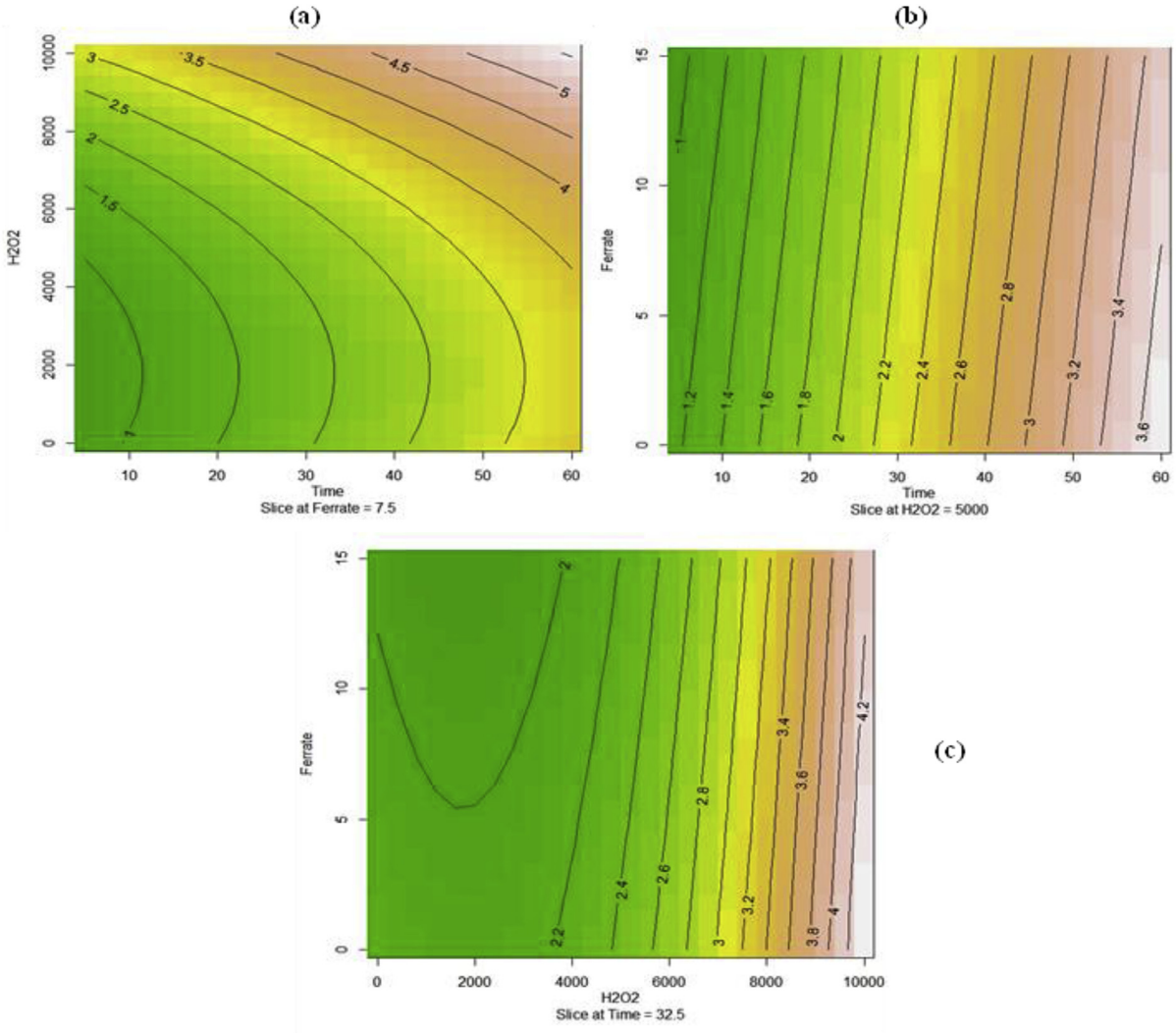

Fig. 2. Response surface plot and its corresponding contour plot of UV/H_2O_2/ferrate process, (a) effect of time and H_2O_2 concentration; (b) effect of time and ferrate concentration; (c) effect of H_2O_2 and ferrate concentrations; and their log inactivation on *B. subtilis* spores.

B. subtilis inactivation. Zhang and Zhou indicated that TiO_2 contributed a significant synergetic effect when combined with UV. The results showed that under various conditions (UV irradiance and TiO_2 concentration) at contact time 600s, about 4–5 log inactivation was achieved. Moreover, Sabeti et al. demonstrated that the combined UV/persulfate process had greatest effect on *B. subtilis* spore inactivation with 4.1 log reduction (Sabeti et al., 2016). The study of Yousefzadeh et al. showed that a maximum disinfection performance (about 4 logs) was achieved in combined UV/nZVI process under deaerated condition in a contact time equal to 60 min and 491 mg/l of nZVI (Yousefzadeh et al., 2018). Based on the comparison of the results of this research with other studies, it is clear that the disinfection efficacy of UV/H_2O_2/ferrate against *B. subtilis* spores at optimum condition is more than other process.

3.3. Effect of pH on inactivation of B. subtilis spores

As highlighted before, for evaluating the effect of pH on inactivation of *B. subtilis* spore, the independent variables were selected at their central point levels and different pH levels from 5 to 11 were applied for the investigation.

The results of this study step indicated that high pH (alkaline condition) for UV/H_2O_2/ferrate process and low pH (acidic condition) for H_2O_2/ferrate process were more suitable to remove *B. subtilis* spores. Fig. 3 represents the effect of pH on *B. subtilis* spore's inactivation. According to the results of UV/H_2O_2/ferrate process, with increasing pH value, the inactivation of *B. subtilis* increased (Fig. 3 a). Since ferrate is stable at alkaline pH, the oxidation potential of environment decreased as pH increased. Furthermore, more precipitation of $Fe(OH)_3$ occurred at low levels of pH; therefore, the effectiveness of UV radiation in the environments with a high level of pH and, consequently, less turbidity, is expected to be better. So, in UV/H_2O_2/ferrate process, the alkaline condition was more effective. On the other hand, in H_2O_2/ferrate process, increasing the inactivation of *B. subtilis* spores at low pH might be the result of higher oxidation potential of components in acidic conditions (Fig. 3 b).

In the case of each process, H_2O_2/ferrate process reduced spores up to 4 log in certain condition and the result of pH showed that at the central point and pH value of 5, about 1.3 log reduction can be achieved while under common circumstances (pH = 8), the reduction of spore was only about 1 log. It is interesting to note that the synergistic effect of UV/H_2O_2/ferrate was more effective and demonstrated the greatest effect on spore inactivation with approximately 5 log reduction. According to the results of changing the pH values, the reduction of spores at a central point and optimal pH of 10.5 was 2.8 log, while at routine pH, it was about 2.5 log.

Many studies have stated that potassium ferrate is unstable in aqueous environments and is decomposed to oxygen (O_2), hydroxide

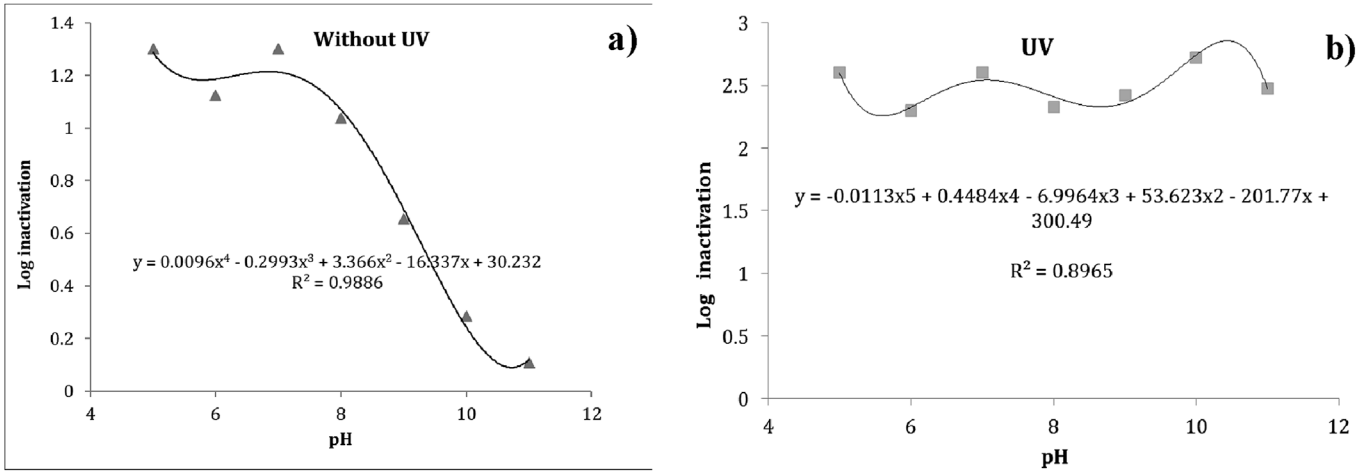

Fig. 3. Impact of pH variations on performance of (a) H_2O_2/ferrate, (b) UV/H_2O_2/ferrate processes for inactivation of *B. subtilis* spore at central point (H_2O_2 concentration = 5000 mg/l, ferrate concentration = 7.5 mg/L, reaction time = 3.5 min).

Table 5
Optimization results of *B. subtilis* spore reduction by combined UV/H_2O_2/ferrate and H_2O_2/ferrate processes.

Process	Time (min)	H_2O_2 concentration (mg/l)	Ferrate concentration (mg/l)	Log inactivation
UV/H_2O_2/ferrate	57	9299	0.4	5.2
H_2O_2/ferrate	60	9846	7.0	4.7

(OH^-), and insoluble hydrous iron oxide [Fe (OH)]. The ionization radiation technique in the presence of Fe(VI) would form Fe(V) (Eq. (6)) in addition to $OH^{\cdot}$. This can enhance the exposure of microorganisms (Sharma, 2002; Kumar and Pandit, 2012).

$$FeO_4^{2-} + e_{aq}^- \rightarrow Fe_4^{3-} \quad (6)$$

From this standpoint, the results of the present study are in agreement with those of other works. The present study indicated that the oxidation potential could be promoted at higher pH (alkaline conditions), hydrogen peroxide concentrations, and contact time. According to the previous studies, in alkaline conditions, Fe(VI) can be converted into Fe(II) and Fe(III), which is quickly deposited and interferes with the penetration of UV light (Cho et al., 2006a). Based on Kazama (1994) study, the reaction of ferrate ion in aqueous solution was complicated and many intermediate compounds might be generated (Kazama, 1994). Because at low pH of UV/H_2O_2/ferrate process, the high production of $Fe(OH)_3$ interfere with UV light; therefore, UV efficiency is reduced. So in this process, the alkaline condition is more effective because of less precipitation of $Fe(OH)_3$ and better efficiency of UV. According to Graham's et al., study (2004), the maximum stability of ferrate appears at pH close to 10 which confirms obtained results of present study. Actually, at higher pH levels ferrate is more stable (Graham et al., 2004). The reaction rates of H_2O_2/ferrate processes are expected to increase at low pH because Fe(VI) has higher oxidation potential at low pH (Jiang and Lloyd, 2002).

3.4. Process optimization

The theoretical optimum values were obtained according to the regression models (Table 3) and by solving Eqs. (7) and (8) in the solver add-Ins in Microsoft Excel (2013) for UV/H_2O_2/ferrate and H_2O_2/ferrate processes, respectively. In this way we have determined the theoretical optimum conditions based on the model. It could be considered as a baseline to investigate the validity of model in future studies which will be done as complimentary study.

$$Y_1 = 2.324 + 1.273\ X_1 + 1.099\ X_2 - 0.1165\ X_3 + 0.0069\ X_1^2 + 0.8473\ X_2^2 \quad (7)$$

$$Y_2 = 0.964438 + 1.0298\ X_1 + 1.1142\ X_2 + 0.01216\ X_3 - 0.8312\ X_2 \times X_3 + 0.3167\ X_1^2 + 0.4523\ X_2^2 \quad (8)$$

where Y_1 and Y_2 are the predicted response (log inactivation of *B. subtilis* spores) by UV/H_2O_2/ferrate and H_2O_2/ferrate processes, respectively; X_1 is the contact time; X_2 is the concentration of H_2O_2; and X_3 is the concentration of ferrate. The optimization results for both processes and pH are presented in Tables 5 and 6.

Based on the theoretical optimal results, the maximum inactivation of *B. subtilis* spores (5.2 log) was predicted after 57 min of treatment by combined UV/H_2O_2/ferrate process with 9299 mg/l of H_2O_2 and 0.4 mg/l of ferrate. According to the coefficients of Table 3 and Eqs. (7) and (8), among the three selected independent variables, the disinfection time (with the coefficient of +1.3) and H_2O_2 concentration (with the coefficient of +1.1) had maximum positive impacts on the disinfection ability of UV/H_2O_2/ferrate and H_2O_2/ferrate processes, respectively. In addition, the impact of ferrate concentrations was insignificant for H_2O_2/ferrate (with the coefficient of +0.01) and it is interesting to note that the ferrate concentration had a negative impact (with the coefficient of −0.12) on UV/H_2O_2/ferrate process.

Many studies have reported that ferrate is stable in alkaline conditions (such as pH of the present study) and its decomposition rate is slow (Cho et al., 2006b). Therefore, its contribution to the inactivation of *B. subtilis* spores by H_2O_2/ferrate is insignificant.

In addition, it should be highlighted that the insignificant negative impact of ferrate on UV/H_2O_2/ferrate could be related to the

Table 6
pH optimization at center point (Time = 32.5 min, H_2O_2 = 5000 mg/l, Ferrate = 7.5 mg/l) for UV/H_2O_2/ferrate and H_2O_2/ferrate processes.

Process	pH	Log inactivation
UV/H_2O_2/ferrate	8.0[a]	2.3 ± 0.3[b]
	10.5	2.8
H_2O_2/ferrate	8.0[a]	1.0 ± 0.4[b]
	5.0	1.3

[a] Initial pH of the study.
[b] Average and standard deviation of inactivation logs at central points (concentration of H_2O_2 = 5000 mg/L, concentration of ferrate = 7.5 mg/l, contact time = 3.5 min).

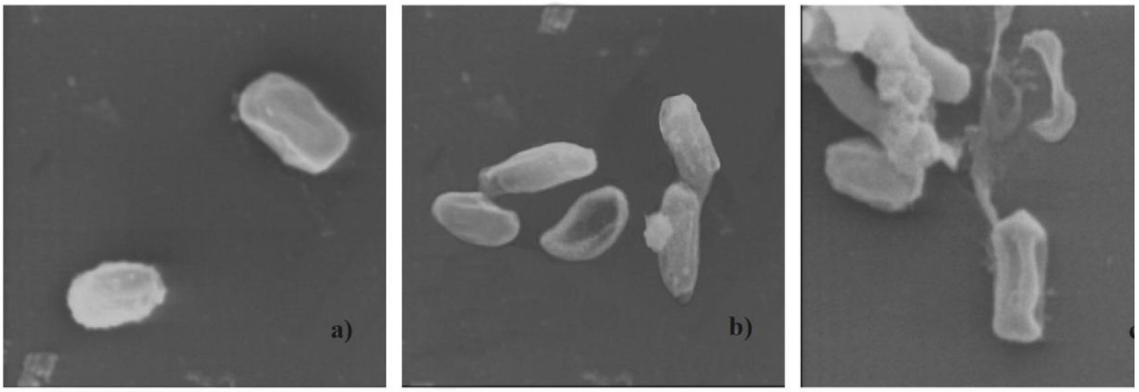

Fig. 4. Scanning electron microscope (SEM) images of *B. subtilis* spores under optimal conditions in different processes a) Unexposed, b) H_2O_2/ferrate and c) UV/H_2O_2/ferrate.

production of $Fe(OH)_3$ from ferrate addition in acidic conditions and its low solubility can result in the precipitation of $Fe(OH)_3$. This precipitation can interfere with and reduce the absorption of UV and, subsequently, reduce the efficacy of the process.

It has been reported that decomposition of both ferrate and H_2O_2 can result in the production of hydroxyl radical ($OH^{\bullet}$) which plays an important role in inactivation of microorganisms. The production of hydroxyl radical from decomposition of Fe(VI) or the intermediates [such as Fe (V) and Fe(IV)] is shown in Eqs (9) and (10) (Cho et al., 2003; Zhao et al., 2017).

$$2FeO_4^{-2} + H_2O \rightarrow 2Fe(OH)_3 + 5[O] \quad (9)$$

$$[O] + H_2O \rightarrow 2HO^{\bullet} \quad (10)$$

3.5. Scanning electron microscopy images

Fig. 4 presents scanning electron microscopy (SEM, ZEISS, DSM960A, Germany) images which represent the damage of spore coat of *B. subtilis* spores under optimized conditions. Fig. 4a is the unexposed *B. subtilis* spores and Fig. 4b and c indicate the effects of combined H_2O_2/ferrate and UV/H_2O_2/ferrate processes which disrupted the spore coat.

A comparison of spores treated with H_2O_2/ferrate (Fig. 4b) and UV/H_2O_2/ferrate (Fig. 4c) revealed that H_2O_2/ferrate process can effect on the spore's structure as pits in the spore's coat as well as transformed and amorphous morphology. In the other hand another process, UV/H_2O_2/ferrate process (Fig. 4c), can hit and pierce the spore coat; therefore, the cellular contents were released into the surrounding surface.

More analysis of SEM images shows that treatment of B. *subtilis* spores with H_2O_2/ferrate process can just resulted in damage to spore coat layers but combined UV/H_2O_2/ferrate with the highest efficiency could disrupt both the spore coat and core.

4. Conclusion

In the present study, RSM was applied to develop, improve, and optimize the operational conditions of the inactivation of *B. subtilis* spores by UV/H_2O_2/ferrate and H_2O_2/ferrate processes. The results of the present study and previous works indicate that the synergistic effect on spores of is useful.

A comparison of spores treated with UV/H_2O_2/ferrate (Fig. 4 b) and control spores (Fig. 4a), can reveal that the coats and cores of *B. subtilis* spores were damaged and ruptured after treatment.

Ultra structural analysis of these SEM images demonstrates a significant reduction after combined UV/H_2O_2/ferrate. It means that this process could disrupt the spore coat and core with the highest efficiency. It is commonly known that hydroxyl radicals are able to enter to the spores and consequently their cellular compositions diffused into the outer perimeter of spores. Therefore, the amorphous morphologies of the spores after the combined UV/H_2O_2/ferrate process were changed.

The present study indicated that the presence of UV had a synergistic effect when applied in the combination of hydrogen peroxide and ferrate. In this work, UV/H_2O_2/ferrate processes were evaluated with CCD design, in which the value of ferrate in the presence of UV/H_2O_2 was not significant for the removal of *B. subtilis* spores. In this design of experiment, it was found that the maximum inactivation of *B. subtilis* spores was related to an increase in the concentration of H_2O_2 and time. According to the analysis and obtained results, the suitability of the second-order model was found to be satisfactory. In addition, the data generated from the quadratic polynomial fitted the response surface well with a high degree of precision and coefficients of determination were 0.99 and 0.96 for R^2 and 0.8 and 0.7 for R^2_{adj} in UV/H_2O_2/ferrate and H_2O_2/ferrate processes, respectively. In this process, $OH^{\bullet}$ radicals had the major role in the enhancement of inactivated of *B. subtilis* spores. More specifically, the greatest synergism was observed when the process of UV/H_2O_2/ferrate was used. Optimization of the processes revealed that UV/H_2O_2/ferrate with 5.15 log of inactivation had the highest amount of removing *B. subtilis* spores. This condition corresponded to 9.23 g/l of H_2O_2 and 0.4 mg/l at the time of 57. Therefore, the results of the present research indicated that UV/H_2O_2/ferrate process is promising and effective for spore inactivation.

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Transparency document

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