Effective electrodisinfection of *Bacillus subtilis* spores with low energy consumption was achieved at room temperature through the synergistic interaction effects of sodium fluoride (NaF) (mg·L⁻¹) and ethanol (mol·L⁻¹), voltage applied (V), pH, and reaction time. *B. subtilis* (10³ spores mL⁻¹) were eliminated with an energy saving of nearly 85% at 0.68 Wh at 4 mg·L⁻¹ NaF and 0.4 mol·L⁻¹ ethanol, using 20 cm² stainless steel electrodes in a batch reactor containing 200 mL of tap water. Lower energy consumption was achieved on the removal of *B. subtilis* spores (10³·mL⁻¹) at pH 7–7.2 after 60 min. No *B. subtilis* spores appeared in colony growth assays following water treatment with 0.05 A and 7 V at neutral pH for this duration. The optimization of treatment parameters allowed for the prediction of *B. subtilis* spore removal in concert with process selectivity according to energy constraints. This study reveals a promising solution to low-cost *B. subtilis* spore removal without generating sludge or changing water quality.
concentrations normally used for drinking water treatment [2]. Current enumeration techniques used to detect C. parvum in water are expensive, cumbersome, and time consuming; therefore, they are not appropriate for routine monitoring [3]. Microbial indicators or surrogates of this resistant protozoan could potentially be used to evaluate the efficiency of disinfection during water treatment. Aerobic spores of B. subtilis, when used as surrogates for C. parvum, have potentially similar resistance to many disinfection processes [4, 5]. Chemical water disinfection problems such as the formation of disinfection by-products, have prompted the search for improved water treatment methods, such as electrochemical water technologies [6, 7]. The electrochemical oxidation process (AOPs) has been recognized as an advanced oxidation process [8]. AOPs, which are based on electrochemistry phenomena, have been applied successfully to the removal of resistant microorganisms from water [9, 10]. In recent years, electrochemical processes have been examined for water disinfection [11, 12]. Electrochemical disinfection can be defined as the extraction of microorganisms using an electric current passed through the water by means of appropriate electrodes. Advantages of the electrodisinfection of water in comparison with other methods include (I) simplicity of the devices, (II) easy automation of the process, (III) no requirement of additional chemicals, and (IV) resulting low current requirement that may allow the use of solar cells or fuel cells as green energy [13, 14]. The method is closely connected to direct and indirect effects of electric current in an electrochemical reactor [15]. The generation of various oxidants such as persulphate or chlorine species, direct oxidation on the electrode surface, inactivation by reactive intermediate products such as hydroxyl radicals, hydrogen peroxide, and ozone, or the electric field effect. The techniques have been proposed for the high bactericidal capacity [16-18]. Its widespread application is hindered by technical problems such as the generation of chlorine by-product species. A number of studies have examined electrolysis, which generates various oxidants including hydrogen peroxide and ozone, as well as free chlorine when chloride ions are present in water, depending on the electrode material, applied voltage, and water chemical composition (i.e., chlorine content) of water. A specific objective of this study was to determine the energy consumption of electrochemical disinfection upon the complete removal of Bacillus subtilis spores from water. This study focused on the effects of NaF and ethanol.

2. MATERIALS AND METHODS

2.1. Microorganism

The bacterial strain used in the present study was B. subtilis ATCC 6633 (Tehran University collection of microorganisms and cell cultures, Iran). The bacteria were maintained on slant nutrient agar at 4°C, and were frozen at −18°C for subsequent short- and long-term maintenance. B. subtilis were reactivated from a frozen stock (10% glycerinated nutrient broth) in a 100 mL flask containing 50 ml tryptic soy broth (TSB, Merck). The sample was incubated at 37°C for about 24 h with continuous aeration. In all experiments, the B. subtilis were inoculated from the stock culture to broth medium at a concentration of 0.5 McFarland standards (~1.5 x 10⁸ per ml). To obtain a spore suspension, bacteria were poured into sterile Erlenmeyer flasks and placed in a water bath at 80°C for 15 min to eliminate
vegetative cells. Sporulation was investigated by optical microscopy using gram staining to confirm the presence of *B. subtilis* spores. Various spores concentrations were prepared by the dilution of *B. subtilis* in drinking water. The pour plate method was used for spores at all concentrations. One hundred microliters of bacterial suspension of *B. subtilis* was spread over the surface of tryptic soy agar (TSA) in 90 mm petri dishes. Following incubation at 37°C for 48 h, the number of spores formed on the agar was counted, and the results were expressed as the mean number of spores per ml. The employed equipment and culture media were sterilized by autoclaving at 121°C for 15 min. The pH was adjusted at the beginning of each experiment by adding NaOH or HCl (0.1 M). The minimal inhibitory concentration (MIC) was determined using the dilution broth method, according to the national committee for clinical laboratory standards (NCCLS) [19]. Briefly, in 12 numbered glass tubes, 10 cc of TSB medium was distributed to each tube. All tubes were sterilized by autoclaving at 121°C for 15 min. MIC was evaluated using 0.2 to 4 M ethanol and tubes were incubated at optimal temperature for 24 and 48 h. After this period the MIC was taken as the concentration of the highest dilution at which the absence of *B. subtilis* growth was observed.

2.2. Electrochemical Experiments

![Figure 1. The electrochemical set-up reactor applied in this study](image)

Experiments were carried out in a single compartment electrochemical reactor (Fig. 1). Two sheets of stainless steel were used as anodic and cathodic material. Experimental runs were conducted by enforcing current density values ranging from 2 to 10 mA/cm², (Atten APS 3005 S-3, China). The
The electrochemical process was run using a batch approach in a 500 mL capacity glass beaker with 300 mL water at ambient temperature. Electrodes were made from steel plate with dimensions $8 \times 4 \times 0.1$ cm (2 cm distance). Each electrode was connected to a DC Power supply with on/off operational switches for controlling the constant current density and voltage. The current density was calculated through the following equation as follows:

$$\text{CD} = \frac{I}{S}$$  \hspace{1cm} (1)

Where $I$ is the current through the solution (A), and $S$ is the area of the electrode (cm$^2$). Throughout the examination, the conductivity and pH of the solution were measured using portable EC and pH meters (Eutech, Singapore). All experiments were performed at pH 7.2. At the experiments' conclusion, the DC power source was switched off and the electrodes were separated from water. During experiments, samples were taken at 15 min interval and cultivated on culture media. All experiments were performed in duplicate. Removal of $B.\ subtilis$ spores ($10^2$–$10^4$ spores·mL$^{-1}$) was examined at 20–100 mA current in the electrochemical disinfection process using ethanol (0.2–0.4 mol·L$^{-1}$) and NaF (1–4 mg·L$^{-1}$) as the additives. The samples were withdrawn every 15 min and transferred onto TSA.

Energy consumption of the process (Wh) is directly related to the cell potential (V), current (A), and time (h) as following:

$$P = V It$$  \hspace{1cm} (2)

3. RESULTS AND DISCUSSION

3.1. Influence of the current density

![Figure 2](image_url)

Figure 2. Inactivation of $B.\ subtilis$ spores as a function of electrolysis time (min) and applied current density. Experimental conditions: batch mode, $10^3$ CFU·mL$^{-1}$, neutral pH, $T = 25^\circ$C, control run without applying supporting electrolytes.
To investigate the efficacy of the electrochemical process on the removal of spores from water, laboratory experiments were conducted under 2, 2.5, and 5 mA·cm\(^{-2}\) electrochemical current density at 15–60 min on water containing 10\(^3\) spores·mL\(^{-1}\). These experiments have shown that at a current density of five mA·cm\(^{-2}\) and a time of 60 min, spore removal takes place (Fig. 2). Haaken et al. reported that increasing current and reaction time resulted in electron transport, and ensuing the generation of hydroxyl ions, ozone, hydrogen peroxide, and free chlorine, finally resulting in disinfection [20].

3.2. Electrochemical Processes Including NaCl

In these experiments, it was shown that increasing current and electrochemical reaction time to eliminate *B. subtilis* spores either increased energy consumption or produced a yellow–brown colored sludge of ferrous and ferric hydroxides in the water.

![Figure 3. Inactivation of *B. subtilis* spores as a function of electrolysis time (min) and applied current density. Experimental conditions: batch mode, 10\(^3\) CFU·mL\(^{-1}\), neutral pH, T = 25°C, with applying supporting electrolytes 4 mg·L\(^{-1}\) NaCl.](image)

The addition of NaCl as a supporting electrolyte in this study was shown to decrease both reaction time and energy consumption. The effect of adding NaCl (0.4\% w/v) on the process were evaluated in the experiments. The results showed that this condition could modify current density from 5 mA·cm\(^{-2}\) to 2.5 mA·cm\(^{-2}\) at 60 min (Fig. 3). Chittoria et al. reported that the high ORP environment created an unbalanced osmolarity between the ion concentration in the solution and that within microorganisms, further damaging membrane structure [21].
3.3. Electrochemical Processes Including Ethanol and NaF in Combination

The effect of 0.4 mol·L\(^{-1}\) ethanol on the kinetics of electrochemical spore removal has been investigated. As shown in Fig. 4, 0.4 mol·L\(^{-1}\) ethanol was able to eliminate spores at a current density of 2.5 mA·cm\(^{-2}\) for 60 min. The obtained results were shown that using 4 mg.L\(^{-1}\) NaF has not effect on the inactivation of Bacillus subtilis spores at electrochemical process. Further experiments were performed to test the effectiveness of the electrochemical process including NaF and ethanol on the inactivation of B. subtilis spores.

![Figure 4](image.png)

**Figure 4.** Inactivation of B. subtilis spores as a function of electrolysis time (min) and applied current density. Experimental conditions: batch mode, \(10^3\) CFU·mL\(^{-1}\), neutral pH, T = 25°C, with applying 0.4 mol·L\(^{-1}\) ethanol.

Spores were readily inactivated at very low current density and contact time, where concentrations of ethanol and NaF were 0.4 mol·L\(^{-1}\) and 0.4 mg·L\(^{-1}\), respectively. In this context, it was shown that the complete inactivation of B. subtilis spores (\(10^3\) CFU·mL\(^{-1}\)) in drinking water could be achieved with 40 min of electrochemical treatment at 5 mA·cm\(^{-2}\) (Fig. 5).

This implies that a NaF concentration of 4 mg·L\(^{-1}\) and an ethanol concentration in the order of 0.4 mol·L\(^{-1}\) are sufficient to electrochemically destroy B. subtilis spores with low energy consumption. Experimental results obtained in this study have shown that the addition NaF and ethanol to an electrochemical process increases the elimination efficiency of spores and saves energy. This can be attributed to the synergistic effect of NaF and ethanol. As shown in Fig. 5, the addition of NaF accompanied by ethanol into an electrochemical system decreases the effective spore removal time, to 50 min and 40 min at current densities of 2.5 mA·cm\(^{-2}\) and 5 mA·cm\(^{-2}\), respectively. To verify the existence of synergism between NaF and ethanol, the synergism parameter was evaluated using the following equation [22]:
\[ S_1 = \frac{1 - I_{1+2}^{+2}}{1 - I_1^{+2}} \]

Where \( I_{1+2} = I_1 + I_2 \); \( I_1 \) and \( I_2 \) are efficiencies of NaF and ethanol for spore removal respectively. \( I_{1+2}^{+2} \) is the measured efficiency for NaF in combination with the ethanol.

Figure 5. Inactivation of *B. subtilis* spores as a function of electrolysis time (min) and applied current density. Experimental conditions: batch mode, \( 10^3 \) CFU·mL\(^{-1} \), neutral pH, \( T = 25^\circ\text{C} \), with applying 0.4 mol·L\(^{-1} \) ethanol and 4 mg·L\(^{-1} \) NaF.

Given that NaF and ethanol into electrochemical process have no effect on each other, then \( S_1 \) is equal to 1. Alternatively, synergistic effects manifest when \( S_1 > 1 \) and antagonistic effects prevail at \( S_1 < 1 \) [23]. Calculated values of the synergism parameter (\( S_1 \)) are displayed in table 1.

<table>
<thead>
<tr>
<th>Synergism parameter (( S_1 ))</th>
<th>Current density (mA·cm(^{-2} ))</th>
<th>Synergism parameter (( S_1 ))</th>
<th>Current density (mA·cm(^{-2} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl and ethanol</td>
<td>2.5</td>
<td>NaF and ethanol</td>
<td>2.5</td>
</tr>
<tr>
<td>0.79</td>
<td></td>
<td>3.04</td>
<td></td>
</tr>
<tr>
<td>0.75</td>
<td>5</td>
<td>2.28</td>
<td>5</td>
</tr>
</tbody>
</table>

Additional results have shown that the addition of NaCl and ethanol to an electrochemical process have antagonistic effect on each other (Table 1); therefore, they have no effect on energy conservation.
As shown in Fig. 6, energy consumption for the combination NaF and ethanol (0.68 Wh) is lower than for all other combinations. Because of its resistance to traditional water disinfection, the *B. subtilis* spore is used as a surrogate of pathogenic waterborne microorganisms such as *Cryptosporidium* and as a quality marker of safe drinking water; therefore, it is of interest to the water supply. Bacterial spores exhibit higher levels of resistance to conventional disinfection than their vegetative cells [24]. Assorted parameters are involved in the persistence of spores, including low water content, the impermeability of the spore cell, high levels of pyridine-2, 6-dicarboxylic acid in their structures, and a thick outer membrane [25]. The DNA of a spore is protected against different types of damages [26]. In recent decades, electrochemical processing as an efficient technology for water disinfection has gained increasing interest. This process could produce free chlorine from the chloride electrolyte, which generates damage to the bacterial outer membrane, penetrating the periplasm, and breaking down the inner membrane, causing degeneration of cytoplasmic proteins [27]. In addition, the electrochemical process can oxidize the microorganisms on the electrode surface. The transformation of low NaCl solutions of 0.05–0.5% into an activated metastable state by electrochemical action produces a solution containing various oxidants, including free chlorine and free radicals, known to possess antimicrobial properties. Electrochemically activated solutions (ECAS) have been shown to have antimicrobial activity against *B. subtilis* spores, and have the potential to be widely adopted within the healthcare environment concern to low-cost initial raw material requirements [28]. The evaluation of a combination of electrochemical processes containing low concentrations of ethanol and NaF for the sterilization of *B. subtilis* spores and comparison with other supporting electrolytes is the main pathway to achieve higher energy savings. Some studies have
reported ethanol with ferric chloride and ethylenediaminetetraacetic acid as sporicidal agent [29]. Another report has investigated different electrodes for energy conservation. These reports have shown that with 3D Cu Ni and stainless steel-based electrodes, energy consumption can be decreased [30]. The present study has demonstrated that by using ethanol and NaF within an electrochemical process, either one can present significant sporicidal efficiency or energy saving of economic feasibility of electrochemical process. Adding the supporting electrolyte NaCl can reduce the voltage requirement for sterilization and generates more free chlorine for microbial destruction, thus decreasing the voltage and time required for spore removal from water. Supporting electrolyte NaF provides more current in the electrochemical process, which is an effective parameter on energy consumption. Ethanol, by entrance into the cellular structure and the denaturation of proteins, can act via synergistic effects.

4. CONCLUSIONS

In the present study, the complete removal of B. subtilis spores from water by an electrochemical process with supporting electrolytes has been investigated. Effects of various parameters including current density, electrochemical time, supporting electrolytes concentration, synergism parameter, and energy consumption on the efficient removal of B. subtilis have been studied. The results of B. subtilis spore removal with an electrochemical process have shown that removal efficiency and energy conservation were enhanced with the increase in electrolyte at 4 mg·L⁻¹ concentration. It was found that the electrolysis time of 90 min reached to 60 min for spore removal at 5 mA·cm⁻² current density. The high effectiveness of supporting electrolytes addition was in 4 mg·L⁻¹ NaF in combination with 0.4 mol·L⁻¹ ethanol in the favor of low energy consumption and thereby low operating cost.

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References


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