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Culturability and Viability of *Salmonella Typhimurium* during Photo-Fenton Process at pH 5.5 under Solar Simulated Irradiation

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ABSTRACT

Culturability and viability techniques such as plate count on solid agar (PC), Most Probable Number (MPN) and Direct Viable Count-Fluorescence *in Situ* Hybridation (DVC FISH) were used to study the inactivation of *Salmonella typhimurium* by photo-Fenton process at pH 5.5. In the presence of only simulated solar irradiation (500 $W \cdot m^{-2}$), *S. typhimurim* showed that both culturability measured by MPN and viability (measured by DVC FISH) underwent just a slight decreasing of 2 and 1 log respectively after 240 min of light exposition while culturability measured by PC did not show any change. Results after 48 h of dark conditions did not reveal re-growth. However, when experiment was carried out in the presence of 2 mg L^{-1} of Fe^{3+} and 20 mg L^{-1} of H_2O_2 and pH 5.5, culturability was strongly affected after 240 min of simulated solar irradiation; nevertheless, viability was only slightly altered (~1 log). During dark period of 48 h changes on culturability and viability were not observed. On the other hand, it was also found that sugar metabolism was affected rather the amino-acids in *S. typhimurium* cells irradiated at different times upon photo-Fenton conditions. These findings might suggest for the first time that photo-Fenton process at pH 5.5 could induce viable but non-culturable state (VBNC) on waterborne *S. typhimurium* and that probably sugar metabolism damage could activate the VBNC state.

Keywords: Photo-Fenton Disinfection; Salmonella Typhimurium Inactivation; VBNC State; Viability and Cultivability

1. Introduction

Photo-Fenton processes which are based in the light induced reaction of ferrous and/or ferric ions with hydrogen peroxide to generate reactive oxygen species such as protonated superoxide (HO_2•) and •OH radical, have recently risen as a promising method to inactivate waterborne bacteria [1-5].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + \bullet OH$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2\bullet + H^+$$

$$Fe(OH)^{2+} + h\upsilon \rightarrow Fe^{2+} + \bullet OH + H^+$$

Recent studies reported that solar photo-Fenton process at near neutral pH (5.5) achieved a complete inactivation of wild *Salmonella sp.*, a pathogen bacterial strain resistant to solar disinfection [6], without re-growth after 72 h of dark storage by simple addition of H_2O_2 in natural waters containing iron at neutral pH [7]. Furthermore at pH 5.5 it was demonstrated that ferric ions undergo a strong interaction with *E. coli* cells avoiding its precipitation and inactivating efficiently the bacteria cells [4]. However all disinfection studies performed with photo-Fenton processes either at acid or near neutral pH have been carried out using plate count methods on solid agar to quantify the microorganisms [2,4,5,7,8]; this procedure could give a false positive since it takes only into account the cell culturability neglecting its viability. Several studies have suggested that when bacteria undergo oxidative stress by the presence of UV light or toxic substances such as radicals, they could enter in the

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transient viable but nonculturable state (VBNC), produced by oxidative stress; VBNC state is a condition where microorganisms lose its capacity to grow in nutritive media [9,10]. Nevertheless, when the oxidative stress ceases, microbial cells can undergo resuscitation where they recover their culturability under specific conditions [10]. This later fact is a key point since some authors have suggested *Salmonella typhimurium* cells could maintain their virulence during VBNC state, and this could imply a serious threat to human health in disinfection processes [11-13].

There are methodologies that can determinate the cell viability. Among them the coupling of Direct Viable count (DVC) with Fluorescence *in situ* Hybridation (FISH) seems to be effective to determinate viability of waterborne bacterial cells [14]. Moreover the coupling of DVC with FISH has successfully been used to determinate viability in *Escherichia coli* cells from natural waters and seems to be a powerful technique to evaluate the viability of waterborne enteric bacteria [15,16].

Herein, for the first time, culturability and viability were measured simultaneously by PC, MPN and DVC-FISH of *S. typhimurim* being exposed to the photo-Fenton processes induced by simulated solar light irradiation at pH 5.5. It is suggested that this photochemical process could induce on the bacterial cells VBNC state avoiding their growing on solid agar and liquid media after 240 min of simulated solar irradiation. This fact is extremely important since it might allow evaluate precisely the ability and limitations of photo-Fenton process to inactive pathogen waterborne bacteria.

2. Materials and Methods

2.1. Photochemical and Photo-Fenton Experiments at pH 5.5

Pyrex glass bottles of 80 mL (3.5 cm in diameter and 10 cm high) were used as batch reactors. 2 mg L^{-1} of Fe^{3+} ($FeSO_4 \cdot 7H_2O$ (Merck-Germany)) and 20 mg L^{-1} of H_2O_2 (Merck-Germany 30% (v/v)) were added to 80 mL of an isotonic solution containing distilled water and NaCl (Sigma-Aldrich Germany) (8.0 g L^{-1}), KCl (Merck Germany) (0.8 g L^{-1}) in each reactor. The initial pH was adjusted by NaOH (Merck Germany) addition until reaching 5.5, not buffer was used. Solar irradiation was simulated by a Hanau Suntest solar simulator (Germany) having a wavelength spectral distribution with about 0.5% of emitted photons <300 nm (UV-C range) and about 7% between 300 and 400 nm (UV-B, A range). The emission spectrum between 400 and 800 nm follows the solar spectrum (**Figure 1**). Light intensity in all experiments was 500 $W \cdot m^{-2}$ and it was monitored with a Kipp & Zonen (CM3) power meter (Omni instruments, Dundee, UK). Pyrex bottles containing inoculated *S. typhimurium* were illuminated in the presence and absence of Fe^{3+} and H_2O_2 during 4 h and samples (1.0 mL) were taken at different time intervals and serial dilutions were performed in saline solution.

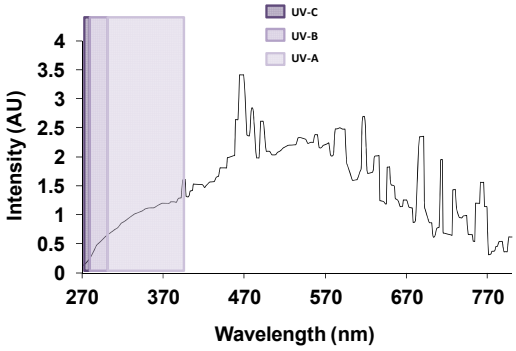

Figure 1. Emission spectrum of solar simulator Hannau Suntest.

The irradiation experiments were performed at room temperature (25˚C) and the temperature of the solution increased up to approximately 30˚C during irradiation. All experiments were carried out in equilibrium agitating at 700 rpm in triplicate and the error bars were added on the graphs.

2.2. Preparation of Salmonella Typhimurium Strain

A strain of *S. typhimurium* ATCC 15490 (Washington DC, USA) was used during these experiments. Strain was preserved in a 30% glycerol solution to avoid membrane rupture during freezing storage. The bacterial reactivation was achieved by inoculating 10 mL of nutrient broth (Oxoid) with 100 µL of bacterial suspension, and then incubating for 18 h at 35˚C. A second inoculum was transferred from this culture to a new tube with 10 mL of nutrient broth and again incubated for 18 h at 35˚C. This second culture was washed by centrifuging at 3000 rpm for 10 min; the supernatant was carefully extracted so the pellet can be suspended and diluted in 10 mL of 0.1% peptonized bi-distillated water (Milli-Q water), agitated and centrifuged again. This procedure was repeated three times, leaving the last cell pellet suspended in 10 mL of 0.1% peptonized bi-distillated water. The final concentration of the washed cell suspension was adjusted according to the McFarland scale #0.5 at 1.5×10^8 CFU mL^{-1}. Consecutive dilutions (10^{-1} - 10^{-4}) of the adjusted cell suspension (1.5×10^8 CFU mL^{-1}) were made in tubes with 9 mL of 0.1% peptonized water (Oxoid), and seeded onto nutrient agar to be incubated for 18 h at 35˚C to establish the initial concentration. This cell suspension was used to inoculate the batch reactors containing the isotonic saline distillated water with Fe^{3+} ($FeSO_4 \cdot 7H_2O$ (Merck-Germany)), and H_2O_2 (Merck-Germany), to initi-

ate irradiation experiments.

2.3. Culturability Measurement of Salmonella Typhimurium by MPN and PC

2.3.1. PC Method

100 µL from each dilution were seeded onto Petri dishes containing Nutrient Agar (Merck-Germany) and incubated at 37˚C ± 2˚C for 24 h to obtain the CFU measuring.

2.3.2. MPN Method

1 mL from each dilution were inoculated onto tubes with 9 mL of lactose broth (Difco) and incubated at 35˚C ± 2˚C for 24 h in order to initiate the presumptive phase. The positives for bacterial growth were then recovered in Rappaport broth (Merck-Germany) before being seeded on Hektoen (Merck-Germany), Xylose, Lysine Desoxycholate agar (XLD agar) (Difco-France) and Nutrient agar (Merck-Germany) to finalize with the confirmatory phase.

2.4. Viability Measurement of Salmonella Typhimurium by DVC FISH

The FISH-DAPI coloration procedure suggested by Amman *et al*. [17] was used for the total cell enumeration, while viable cells were enumerated using the DVC-FISH method proposed by Kogure *et al*. [18] and Regnault *et al*. [19] with the following modifications.

2.4.1. DVC-FISH

The remaining 8.8 mL sample in each dilution tube were treated with Nalidixic acid (40 µg mL^{-1}) and 1 mL of lactose broth (Difco-France) and yeast extract (Difco-France), then incubated in the dark for 18 h at 35 ± 2˚C. Cells were harvested by centrifugation at 5000 g. Depending on the expected concentration, 100 or 300 µL were re-suspended in 10 x phosphate buffered saline (PBS) solution to reach 1 mL and fixated with a *para*-formaldehyde fixation buffer 30% (v/v), stored overnight at 4˚C, and harvested by centrifugation at 1000 g for 10 min. The pellets were washed with PBS solution. This washing process was repeated two times to ensure the complete removal of *p*-formaldehyde, and the final pellets were re-suspended in 500 µL of 1 × PBS and 500 µL of ethanol 100%. 10 µL of the resulting cell suspension was transferred to each well of a 8-well slide, previously covered with gelatine (0.1% w/v) and $KCr(SO_4)_2$ (Merck-Germany) (0.01% w/v). The loaded slides were dehydrated with ethanol and incubated for 2 h in the dark with 10 µL of hybridization solution pre-warmed at 45˚C and containing 24 ng·$\mu L^{-1}$of *S. typhimurium* probe [16,20], marked with 5' Cy3 (prepared by Microsynth GmbH Switzerland). Finally, cells were washed with a washing solution at 45˚C and then rinsed with distilled water.

2.4.2. DAPI

After hybridization, all samples were washed in distilled water and stained with 9 µL of DAPI (Organic Research-Ireland) (4',6'-diamidino-2-phenylindole) 0.0001% (w/v) for 10 minutes, washed again in distilled water and dried (to aid cell localization, slides were cover with anti-fade citi-fluor solution). Microscopic cell count (DAPI) and oligonucleotide probe-positive count (Cy3) were performed using a Nikon Eclipse E800 microscope (Japan) equipped with a specific DAPI filter and G-2A filter sets. A minimum of 20 view squares were enumerated for each well; two wells were examined in total.

2.5. Assays of Growing in Mineral Enriched Media

Four tubes containing 20 mL of mineral media containing distilled water and NaCl (Sigma-Aldrich Germany) (8.0 g·L^{-1}), KCl (Merck Germany) (0.8 g·L^{-1}) and 20% of Glucose (Merck Germany) or Peptone (DIFCO UK), respectively, were inoculated with 1 mL of irradiated bacteria. The turbidity at 650 nm was measured after 18, 24, and 96 h of incubation and the increase in 0.1 or more turbidity units was reported as positive growth.

3. Results and Discussion

3.1. Effect of Simulated Solar Light Arradiation on Culturability and Viability of *S. Typhimurium* Cells in Absence of Ferric Ions and H_2O_2

The initial *S. typhimurium* concentration before irradiation events, evaluated with DVC FISH and MPN methods, achieved concentrations two logs higher than the PC method (**Figure 2**). Although there is no difference between the nutrient composition of a liquid and a solid culture media, in practice the culture broths have a higher

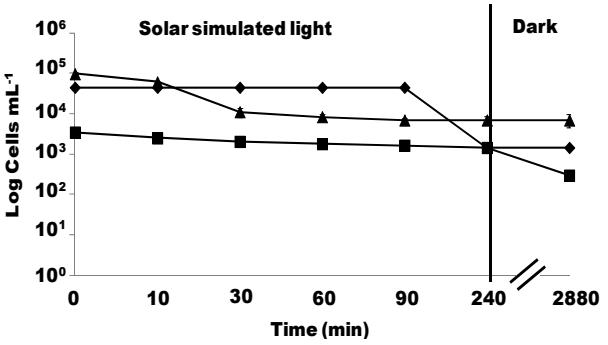

Figure 2. Inactivation of *S. typhimurium* by simulated solar light (500 W·m^{-2}) at pH 5.5 followed by -■-PC, -◆-MPN and -▲-DVC FISH.

rate of cell recovery. In solid media (PC method) the bacteria growing is limited by the availability of water and nutrients close to the colony's growing area while the liquid media (MPN and Nalidixic media) ensures constant and direct contact of cell with nutrients. This could explain why DVC-FISH and MPN gave initial concentrations higher than PC.

On the other hand, results obtained by MPN and PC (**Figure 2**) revealed that after 240 min of solar simulated irradiation, the former method showed that *Salmonella* population underwent a decreasing of almost 2 logs while the later did not show any decreasing of bacterial concentration. Furthermore, DVC FISH viability count underwent a slight decreasing of its initial concentration (~1 log) after of 240 min of solar simulated irradiation. The experiments were followed by 48 h in dark conditions and the results showed that DVC FISH and MPN did not undergo neither decreasing nor re-growth on the bacterial population. However PC method showed a slight decreasing of almost 1 log probably due to the fact that under these conditions weakened microorganisms (by UV irradiation) could undergo an additional stress by the lack of nutrients. This decreasing of *E. coli* population after a photocatalytic treatment (in presence of TiO_2 and solar simulated light) under dark conditions was already observed by Rincon and Pulgarin [21] and referred as post-irradiation events.

In summary, the three methods used in this study (DVC FISH, MPN and PC) revealed that direct solar simulated irradiation had slight negative effect on the *S. typhimurium* population. It is well known that UV-C component (220 - 280 nm) of UV light is the most lethal to the microorganisms since it might produce chemical changes on the DNA components [22]. Solar simulator emits few UV-C light irradiation (**Figure 1**) and this fact could explain why light irradiation did not affect in a larger extent of the initial concentration of *S. typhimurium*. However, the UV-A (320 - 400 nm) is emitted by the solar simulator and some studies have argued that this UV component could affect the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and could also produce partial degradation of iron-containing proteins, releasing Fe (II) ions into the cell [23, 24]. Thus, only the presence of UV-A light could generate a bacteria weakening by diminishing its antioxidant capacity without producing a lethal damage.

3.2. Effect of Photo-Fenton Process at near Neutral pH on Culturability and Viability of *S. typhimurium* Cells

Figure 3 shows that culturability measured by MPN and PC was strongly affected by photo-Fenton process at pH 5.5. Results obtained by MPN revealed a diminution of 4 logs after 240 min of simulated solar irradiation and PC

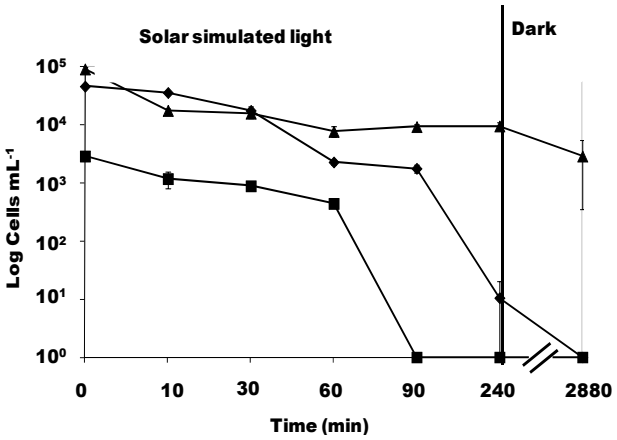

Figure 3. Inactivation of *S. typhimurium* by simulated solar light (500 $W \cdot m^{-2}$) in presence of 2 $mg \cdot L^{-1}$) of Fe^{3+} and 20 $mg \cdot L^{-1}$ of H_2O_2 at pH 5.5 followed by -■-PC, -◆-MPN and -▲-DVC FISH.

results showed a total loss of culturability after 90 min of irradiation. In contrast, viability followed by DVC FISH underwent a slight decreasing of around 1 log. In dark conditions no re-growth or loss of viability was observed.

A previous study [4] revealed that neither H_2O_2 nor ferric ions induced a strong toxic effect on *E. coli* cells and in this study was found the same trends (Data not shown).

Photo-Fenton processes are often carried out at acidic pH in order to avoid iron precipitation. However, in aqueous media and at pH close to 5, iron soluble species such as $Fe(OH)_2^+$ exist and could lead to the formation of high oxidative radicals [3,25]. Furthermore, a recent study reported that ferric ions at pH 5.5 are strongly adsorbed by *E. coli* cells preventing its precipitation [4]. Although several studies have reported photo-Fenton process as a promising technology to inactivate waterborne bacteria [2,4,5,7,8], these have only followed culturability on solid media. Until now, there are not studies regarding bacterial viability during and after the photo-Fenton process.

When bacteria cells among them *S. typhimurium* undergo oxidative stress caused by the presence of external or internal toxic substances or by the presence of oxidative radicals, they can enter in a state called VBNC that seems to be an adaptive response aimed to survive in adverse conditions [10,26], where the metabolic processes decrease avoiding that bacteria can grow on solid or liquid media. This fact could explain why during photo-Fenton process, *S. typhimurium* cells showed a strong loss of culturability while viability remains almost unaltered. Recently, it was suggested that iron cations could interact strongly with negatively charged *E. coli* membranes [4] (isoelectric point ~ 4.3 [27]) at near neutral pH. Once the photo-Fenton process at pH 5.5 (with iron cations and H_2O_2 on the outer membrane) induces

the formation of •OH with a higher oxidative power (2.8 eV vs NHE) but shorter lifetime (ns); these radicals might attack only the *S. typhimurium* outer membrane inducing lipid peroxidation (LPO) and causing permeability alterations [28]. Furthermore, it has been reported that H_2O_2 might also induce changes on motility and catalase activity in *S. typhimurium* affecting its ability to defend against the deleterious reactions of reactive oxygen species (ROS) or oxidative attack [29]. Moreover, some studies have reported that in bacteria such as *Vibrio vulnificus* and *E. coli* 0157:*H* the decreasing of catalase activity and the presence of H_2O_2 would be narrowly linked to the VBNC state induction [30,31].

In order to evaluate the recovery of *S. typhimurium* after different exposure times during the photo-Fenton treatment, irradiated cells where transferred onto two different solutions of mineral media enriched with either glucose or peptone, measuring optical density (O.D.) after 18, 24 and 96 h of dark storage (**Table 1**). Results show that *S. typhimurium* grows in both media before photochemical treatment since cells have not undergone any damage. This agrees with previous studies [32].

After 1 h of photo-Fenton treatment and 18 h in dark storage, *S. typhimurium* cells did not show any growth in glucose-enriched media, but then at 24 h and 96 h in dark storage re-growth was observed. With 2 h and 3 h of photo-Fenton treatment, bacterial re-growth was observed only at 96 h in dark storage. Finally after 4 h of photochemical treatment *S. typhimurium* did not show any re-growth at 18 h, 24 h or 96 h in dark storage.

When using peptone-enriched mineral media for cell recovery, different results were obtained. Bacterial cells grew after 1, 2 and 3 h of photo-Fenton treatment during 18 and 24 h in dark storage. However after 4 h of photochemical treatment media did not showed any growth. These results imply that photo-Fenton treatment might induce damages on sugar metabolism rather than amino-acidic metabolism (peptone is conformed mainly by amino-acids) in *S. typhimurium* cells.

Herein is suggested that the UV-A irradiation and H_2O_2 might affect the catalase activity of *S. typhimurium*, weakening its anti-oxidant capacity. Furthermore, the attack of •OH radicals might produce damages mainly on the outer membrane by lipid peroxidation (LPO) or oxidizing proteins responsible of sugar metabolism (SMP). This could be the trigger alarm inside the microorganism to make it enter into the VBNC state (**Figure 4**). Probably, *S. typhimurium* cells in VBNC state could maintain its infective capacity after 4 h of near neutral photo-Fenton treatment under solar simulated irradiation. This fact should represent a high risk for human health, since non-culturable cells of pathogenic bacteria can retain substantial physiological activity, including the capacity to synthesize toxins being able to induce disease [30,33].

In spite of these results, further experiments are necessary in order to find the experimental conditions where photo-Fenton processes can reach viability loss and grant the bacterial dead. Furthermore, studies of molecular biology must be carried out to understand what cell targets are attacked during the photo-Fenton process.

Table 1. Cell grow ability under mineral media enriched either glucose or peptone monitored after photo-Fenton treatment during dark storage.

Irradiation time (h)	Glucose			Peptone		
	Dark storage (h)					
	18	24	96	18	24	96
0	+	+	+	+	+	+
1	-	-	+	+	+	+
2	-	-	+	+	+	+
3	-	-	+	+	+	+
4	-	-	-	-	-	-

4. Conclusions

Culturability and viability of *S. typhimurium* cells during photo-Fenton process at pH 5.5 induced by solar simulator light was followed through culturability and viability techniques such as PC, MPN and DVC FISH.

Results showed that under our experimental conditions photo-Fenton process strongly affects the *S. typhimurium* cells culturability rather its viability. Thus, this photochemical process might induce a VBNC state probably due to a combined effect of UV-A light, H_2O_2 induced-weakening of catalase activity, and the oxidative attack of photo-induced •OH radicals on the outer bacterial membrane causing lipid peroxidation and loss of permeability. Besides, it was found that glucose metabolism rather the amino acid was affected by photo-Fenton processes at near neutral pH.

The presence of *S. Typhimurium* in VBNC state after near neutral photo-Fenton process induced by solar light might be a serious issue since this would demonstrate that this photo-Fenton process might cause a bacterio-

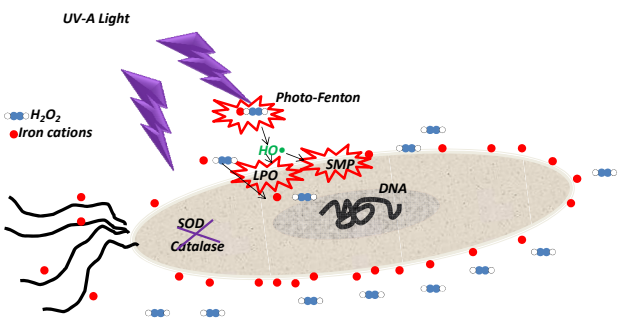

Figure 4. Suggested mechanism of VBNC state induced by photo-Fenton at pH 5.5 on *S. typhimurium* cells.

static but not a bactericidal effect. However in spite of these results, further experiments are necessary to under-stand the true effect of photo-Fenton processes on wa-terborne bacteria inactivation and find the experimental conditions where viability is also affected since thus the possibility of re-growing after photo-Fenton processes would be reduced.

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