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Bacterial inactivation in wastewater using UV/H_2O_2 advanced oxidation

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The antibiotic resistant bacteria have been spread from the environment to human through the water and the inactivation processes may be a possible way to reduce this type of threat. The main objective of this work is to investigate inactivation of *E. coli* present in wastewater employing direct UV andadvanced UV/H_2O_2 process. Bacterial inactivation both in real wastewater as well as synthetic wastewater was also investigated. Direct photolysis at 253.7 nm showed only 61% inactivation when initial bacterial concentration was 1.7×10^7 CFU/mL. In case of UV/H_2O_2, about 99% bacterial inactivation was observed at 240 min treatment. It is concluded that during the advanced oxidation process, the chemical compound hydrogen peroxide (H_2O_2) absorbs UV light andhydroxyl radicals are produced by photolysis. These hydroxyl radicals are strong oxidizing agents and help to inactivate the bacteria.

Keywords: Antibiotic resistance, *E. coli*, UV, H_2O_2, Wastewater.

Introduction

Antibiotics are the most important group of drugs in today's pharmacy. These antibiotics are mostly used for human, veterinary and agriculture field. For the huge consumption of antibiotics in various fields, these materials are tremendously discharged into environment from different anthropogenic sources[1–3]. Thus, in recent years; the great concerns keep rising about potential impact of the remaining part of antibiotics in the aquatic environment[4–6]. Now-a-days, the antibiotics can be identified in surface water excluding the original site in the mountainous region where the streams or rivers not going through the agricultural and urban areas[7]. Some antibiotics can be observed even in groundwater at a depth of more than 10 m[8,9].

The antibiotics are considered as chemical pollutants due to its harmful effects on the environment. These are also responsible for the dispersion of antibiotic resistant bacteria (ARB leading to health problem in human beings and animals[6,10]). These bacteria may be transferred from environment to human through different contact directly or indirectly[11]. Water is the main media for antibiotic resistance selection and the antibiotic resistance bacteria are diffused from the environment to humans via water cycle[12], particularly those areas where the effect of anthropogenic activity is high[13]. Therefore, the environmental contamination and the associated human health risk via food chain has been considered as a severe public health issue as stated by World Health Organization (WHO) which recognized the antibiotic resistance development is one of the most global threats to the human community[14].

Unfortunately, most treatment options such as conventional techniques (filtration, biological processes, sedimentation and flocculation), membrane techniques, adsorption, and combined methods[15], coagulation[16], and UV radiation[15] considered only disinfection (killing of the bacteria) or removal of antibiotic. These, however, do not remove the resistant bacteria[17–19] and once the antibiotic resistance genes are localized in plasmid or transposons via antibiotic resistance bacteria they have the potential to persist in environmental matrices or originate resistance in other bacteria[20]. Similarly, if these resistance bacteria are not removed from the final effluents, they can cause resistance to bacteria residing in normal gut flora of the organism in the water. Therefore, it is an urgent necessity to focus on removal strategies

of antibiotics along with antibiotic resistance bacteria by advanced processes. McKinney and Pruden (2012) observed that UV irradiation is directly absorbed by the DNA of the bacteria and this inactivates antibiotic resistance bacteria[21]. Few literatures have studied about the ARB (*Enterococci* sp.) inactivation by solar Fenton[22] and various photocatalysis processes[23]. Unfortunately, there is little work which studied about the effect of UV light and H_2O_2 doses separately and effect of UV/H_2O_2 on inactivation of *E. coli* in the wastewater at the same time exposure. Accordingly, the objective of this work is to investigate inactivation of bacteria present in wastewater by UV and UV/H_2O_2 advanced oxidation process (AOP). AOPs may remove microorganisms and bacteria by the production of strong oxidizing agent i.e. hydroxyl radicals[24–26] and can also destroy the products generated from lyse bacterial cell[27]. The bacteria chosen for this study was *E. coli,* a Gram-negative bacteria and present in the normal gastrointestinal function of the humans and animals. Particularly, the effect of UV/H_2O_2 on *E. coli* inactivation from wastewater was investigated.

Materials and methods

(A) Reagents:

All analytical grade quality reagents were used for this study. MUG Agar and lactose broth were received from (Hi-Media, India); whereas 30% H_2O_2 and the antibiotics ciprofloxacin (CIP) were obtained from Merck, Germany and Frank Ross, IIEST, Shibpur, Howrah respectively. All solution was prepared by using steriled or autoclaved (at 121°C and 15 lb pressure for 15 min) water.

(B) Wastewater samples:

The wastewater samples were collected from the municipal drain of College Ghat near Shibpur, Howrah. The sample was collected in 120 mL plastic bottle, filtered using commercial filter paper and was stored in refrigerator at 4°C till further use.

(C) Isolation and culture of resistant E. coli:

Antibiotic resistant *E. coli* was isolated from the wastewater using selective MUG Agar (Peptic digest of animal tissue - 5.000 g/L, sodium chloride - 5.000 g/L, beef extract - 1.500 g/L, yeast extract - 1.500 g/L, 4-methylumbelliferyl β-D-glucuronide - 0.100 g/L, agar - 15.000 g/L)[28]. Briefly, 10 mL of MUG Agar solution was prepared by diluting the agar into steriled water and was autoclaved at 15 lb pressure and 121°C for 15 min respectively. Then, the molten Agar solution was poured onto the petriplate and keep in the room temperature to get solidify. After solidification, 100 μL of wastewater were added to the solidified MUG agar solution and the petriplate was kept in an incubator for 24 h at 37°C.

After incubation, a few colonies of *E. coli* were randomly picked up from the plate and dissolved to 5 ml of lactose broth (Peptone - 5 g/L, HM Peptone Beef extract - 3 g/L, Lactose - 5 g/L) solution and incubated overnight at 37°C. Thus, a resistant *E. coli* culture was prepared on Lactose broth medium. Then the culture of *E. coli* was frozen at -20°C for further use.

(D) Studying antibiotic resistance of the isolated E. coli:

The bioassay studies were performed to find the resistance in *E. coli* against the antibiotic CIP by broth dilution method[29]. A loopful of each bacterial isolate was dissolved in 0.9 sterile saline solutions for the preparation of inocula and then the turbidity of the solution was measured. The turbidity was compared with 0.5 McFarland standard solutions and suitably re-adjusted for uniform cell count, if required. The obtained suspension was used for antibiotic susceptibility testing by serial dilution method. Stock solutions (2 mg/L) of the antibiotic were prepared and it was serially diluted in different test tubes in 2 times dilution. 100 μL inoculum and 100 μL antibiotic standard solutions were added to 96 well micro plates. Then the micro plates were incubated for 24 h at 35°C. After incubation, the optical density of the prepared samples was measured by using microplate reader (Thermo Scientific, Model - multiscan GO) and growth inhibition percentage was calculated. Then the *E. coli* growth inhibition was calculated by converting the Optical density using eq. (1).

$$I\% = \left(\frac{OD_{625,pos} - OD_{625,exp}}{OD_{625,pos} - OD_{625,neg}} \right) \times 100\% \quad (1)$$

In eq. (1), I = percentage of inhibition; OD_{625} = optical density at 625 nm. The subscripts 'exp', 'pos' and 'neg' represent the experimental samples, positive growth control and negative growth control respectively.

The data of the *E. coli* growth inhibition were fitted to the Hill's equation[29] to find the 50% inhibition concentration and Hill's slope.

$$I\% = I_{min} + \frac{I_{max} - I_{min}}{\left[1 + \left(\frac{IC_{50}}{C}\right)^{H}\right]} \quad (2)$$

In eq. (2), I_{max} = maximum bacterial growth inhibition; I_{min} = minimum bacterial growth inhibition and IC_{50} = CIP concentration corresponding to 50% inhibition of *E. coli* growth. The constants, *C* and *H* represent the concentration of CIP and Hill slope respectively.

(E) Analytical method:

Bacterial concentration in synthetic wastewater and real wastewater was determined by McFarland method[30] and another is plate count method[28] respectively. In McFarland method, a calibration curve was prepared for bacterial concentration (no. of *E. coli* in CFU/mL) with absorbance of McFarland standards at 625 nm (Fig. 1). A McFarland Standard is a fine precipitation of barium sulfate prepared by the mixing of two chemicals such as barium chloride and sulfuric acid. The absorbance of the cultured sample obtained was interpolated in the calibration curve and the bacterial concentration was measured.

The plate count method is based on the bacterial growth in forms of colonies on nutrient medium. The bacterial colonies become visible normally to the opened eye and the number of bacterial colonies on a petriplate can be counted. The collected wastewater samples were filtered using commercial filter papers to remove suspended solids. Thereafter, the filtrate was suitably diluted by sterile water by serial dilution technique. Then 100 μL each diluted sample was transferred to the sterile selective MUG Agar solution on the petri dishes to culture *E. coli* strains respectively and the petri dishes were incubated overnight at 37°C. After 24 h of incubation at 37°C, colonies were counted and the no. of bacteria was calculated as CFU/mL by plate count method.

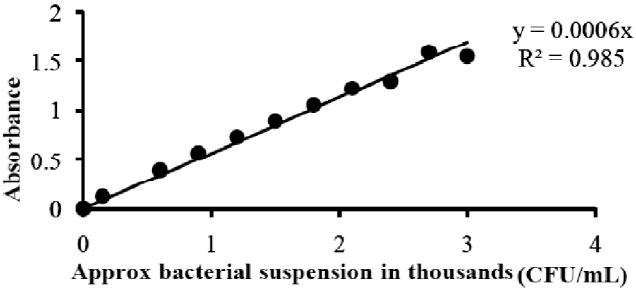

Fig. 1. Calibration curve for bacterial concentration.

(F) Inactivation of E. coli using UV and UV/H_2O_2 process:

The UV and UV/H_2O_2 experiments were performed in UV reactor in batch mode. The UV reactor was procured from M/s. Lab Tree, India. The reactor is fitted with eight UV tubes (peak wavelength of 253.7 nm). The UV reactor has been designed with a glossy stainless steel reflector, in which the eight UV tubes were fitted in a heavy metal enclosure. Potassium ferrioxalate actinometry method was used to measure photon flux of the reactor[31]. The photon flux was determined to be $1.9(\pm 0.1)\times 10^{-4}$ Einstein/L-min and fluence of the system was calculated to be 113(±5.7) mJ/cm^2-min.

100 mL of synthetic and real wastewater samples were used in each of the batch study. For the UV and UV/H_2O_2 experiments, the samples were placed in a 250 mL of quartz beaker within the UV reactor. The upper and lower surface of quartz beaker was covered and the samples were treated for 6 h. Then samples were collected at different time intervals and analyzed for remaining bacterial concentration. The first order reaction for bacteria inactivation was represented by the following equation[32].

$$\ln \frac{C}{C_0} = -kt \quad (3)$$

In the above equation, C_0 = initial bacterial concentration; *C* = concentration of bacteria at time *t*; *k* = rate constant value of first order reaction and *t* = reaction time. For the UV/H_2O_2 experiments, the effects of different peroxide doses were also studied. The hydrogen peroxide doses were 2.5, 10, 20, 40, 50 and 60 mg/L. The pH of solutions was 7.5. During the treatment, 2 mL of samples have taken at time intervals from 0 to 240 min of irradiation and analyzed for remaining bacterial concentration. The experimental data were fitted in the above-mentioned kinetic equation (eq. (3)).

Results and discussion

(A) Determination IC_{50} of E. coli:

The municipal wastewater samples for the bioassay studies were collected from College Ghat drain, Shibpur, Howrah. Firstly the *E. coli* was isolated from the samples using agar medium. Then the isolate was cultured in lactose broth solution and bioassay was performed using the cultured bacteria. The bioassay results were then fitted to Hill's inhibition model to obtain the required IC_{50} values. Following Fig. 2 illustrates the inhibition profile of *E. coli* isolated from municipal wastewater. The IC_{50} and H values were obtained 1717 μg/L and 0.4704 respectively. The IC_{50} value for pure *E. coli* was measured and found to be 25 μg/L. Hence, *E. coli* in the wastewater samples of Howrah has developed 69 times resistances against CIP.

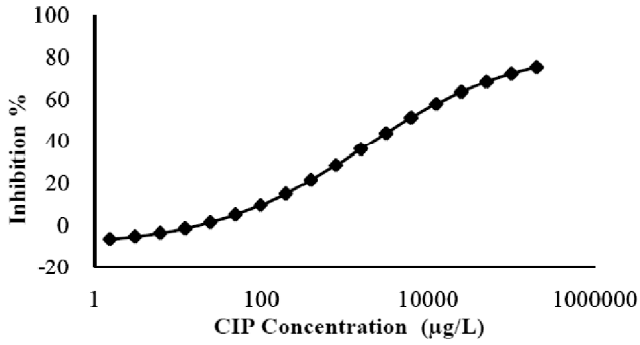

Fig. 2. Inhibition profile of *E. coli* at different CIP concentration.

(B) Inactivation of E. coli in synthetic wastewater:

Synthetic wastewater was prepared in laboratory by diluting the cultured bacteria (isolated from municipal wastewater) in distilled water. The prepared synthetic wastewater was treated by UVand UV/H_2O_2 process as discussed below.

(B.1) Inactivation E. coli in synthetic wastewater by UV light:

Direct UV experiments were conducted using initial *E. coli* concentration of 1.7×10^7 CFU/mL at a pH of 7.5. Samples were withdrawn at regular time intervals (30, 60, 90, 120, 150, 180 and 240 min) and analyzed for remaining bacterial concentration by McFarland method. It was seen that the *E. coli* concentration diminished from 1.7×10^7 CFU/mL to 6.7×10^6 CFU/mL after 240 min. The inactivation of *E. coli* was found to be approximately 61% (Fig. 3). Kinetic analysis

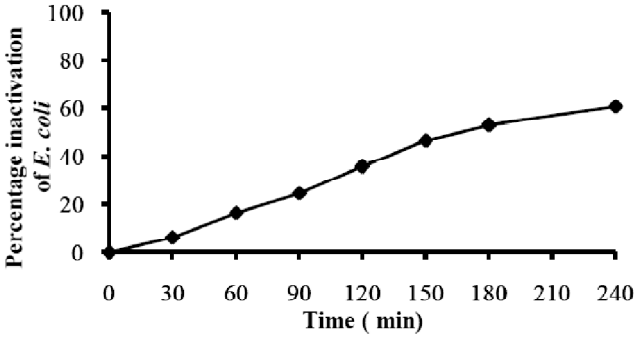

Fig. 3. Inactivation of *E. coli* by UV (253.7) light.

was done to determine the rate of reaction at which UV degradation occured. Rate constant value was determined to be 0.0039 min^{-1} which was obtained from the Fig. 4. The inactivation of *E. coli* is due to direct absorption of UV light by the nucleic acid such as DNA of the bacteria and damage of DNA and some of the essential proteins and lipids of the bacteria[33,34].

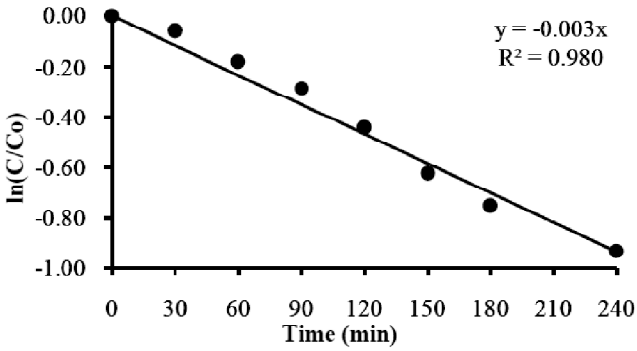

Fig. 4. First order kinetics model of inactivation of *E. coli* by UV (253.7) light.

(B.2) Inactivation of resistance E. coli in synthetic wastewater by UV/H_2O_2 process:

Despite the above positive results obtained during UV radiation alone, it was expected that a UV based AOP i.e. UV-H_2O_2 would also be more effective for inactivation of *E. coli*. Therefore, the synthetic wastewater was prepared by diluting the *E. coli* culture in 100 mL steriled (15 lb pressured for 15 min) water and mix vigorously. Then, 100 mL of synthetic wastewater were irradiated under UV with different doses of H_2O_2 (2.5, 5 and 10 mg/L) at a pH of 7.5. This helps to optimize peroxide dose for certain degree of *E. coli* inactivation. Initial *E. coli* concentration was 1.7×10^7 CFU/mL which

was decreased to 1.0×10^6 CFU/mL, 1.0×10^5 CFU/mL and 1.2×10^5 CFU/mL at a peroxide concentration of 2.5, 5 and 10 mg/L at 240, 180 and 90 min respectively. It was noticed that 94%, 99% and 99% inactivation of *E. coli* was achieved at a H_2O_2 dose of 2.5, 5 and 10 mg/L at 240, 180 and 90 min respectively (Fig. 5). A high percentage (close to100%) inactivation of *E. coli* was observed at 5 and 10 mg/L H_2O_2 dose after 150 and 60 min. These results indicate that there was a particular pattern between the percentages inactivation of *E. coli* with H_2O_2 doses. It was found that with increasing the peroxide doses, the complete inactivation of *E. coli* took place at different time interval up to 4 h. It was interesting to note that the inactivation of *E. coli* was very less in presence of peroxide in dark condition. The maximum of 5% inactivation of *E. coli* was achieved after 240 min at a constant H_2O_2 concentration of 60 mg/L.

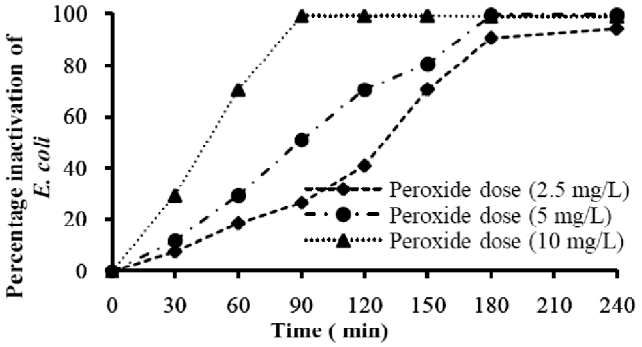

Fig. 5. Inactivation of *E. coli* in synthetic wastewater by UV/H_2O_2.

Kinetics analysis was made to determine reaction rate of UV/H_2O_2 process. It was noticed that the reaction followed first order rate reaction model and the rate constants for 2.5, 5 and 10 mg/L of H_2O_2 were 0.0096, 0.0097 and 0.0186 min^{-1} respectively; which was increased gradually with increase in concentration of H_2O_2. From the kinetic values of the first order kinetics model (0.0096 min^{-1} to 0.0186 min^{-1}), it was apparently showed that *E. coli* was affected by the increasing H_2O_2 concentration at different rate[35].

The hydroxyl radical's activities in decreasing the bacterial concentration may be described similar to UV disinfection mechanism. Inactivation of bacteria by UV/H_2O_2 is due to HO• radicals were produced by the photolytic reaction of UV light and these radicals attack to the cell wall of the bacteria[36,37]. It has been reported that direct attack, oxidation, and distraction of the cell membrane and wall by UV and H_2O_2 are main reasons for disintegration of the cell, resulting in bacterial inactivation[38].

(C) Inactivation of resistant E. coli in real wastewater by UV/H_2O_2 process:

The inactivation of antibiotic resistant *E. coli* in real wastewater was studied under advanced oxidation process using UV light and hydrogen peroxide. The wastewater was collected from College Ghat, Shibpur, Howrah, West Bengal and it was also observed that the antibiotic resistance *E. coli* in the wastewater was 69 times resistances against CIP. 100 mL of wastewater was treated by UV at different at different peroxide doses of 2.5, 40, 50 and 60 mg/L at pH 7.5. During the treatment of real wastewater with UV and different peroxide doses, the initial *E. coli* concentration was found to be 1.7×10^7 CFU/mL. Under this condition, maximum 86 percentage of inactivation of *E. coli* was achieved at 60 mg/L H_2O_2 dose at 240 min (Fig. 6). At the irradiation time of 240 min, the percentage inactivation of *E. coli* was found to be 65, 67 and 76% for other hydrogen peroxide doses of 2.5, 40 and 50 mg/L respectively.

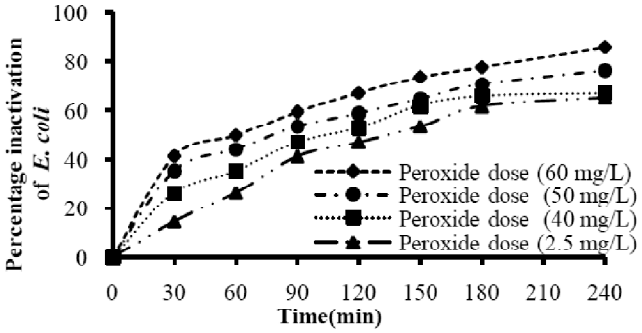

Fig. 6. Inactivation of *E. coli* in real wastewater by UV- H_2O_2 process.

The rate constants for different concentration of H_2O_2 were determined by using first order kinetic model. The values of rate constants (k) and R^2 for different H_2O_2 concentration were summarized as follows: k = 0.0049 min^{-1}, R^2 = 0.9683 for 2.5 mg/L H_2O_2; k = 0.0065 min^{-1}, R^2 = 0.9761 for 40 mg/L H_2O_2; k = 0.0068 min^{-1}, R^2 = 0.9008 for 50 mg/L H_2O_2; and k = 0.0087 min^{-1}, R^2 = 0.9454 for 60 mg/L H_2O_2. The rate constants were found to increase with the increase in hydrogen peroxide concentration. From the above results, it was indicated that the hydroxyl radical produce from UV/

H_2O_2 process was the driving force behind higher *E. coli* inactivation. This deleterious and fast deterioration in the bacterial population can be recognized by the production of $HO^{\bullet}$ from H_2O_2 by photolysis (eq. (4)).

$$H_2O_2 + h\nu \rightarrow 2OH^{\bullet} \quad (4)$$

Moreover, photons reaching *E. coli* can change their cell functional structure and damage their DNA and the hydroxyls radical destroy their intracellular activity and inactivate the bacteria[39]. The percentage removal in synthetic wastewater is higher (about 14%) than in real wastewater, it might be due to the presence of some ions such as bicarbonate (HCO_3^-) in real wastewater. These bicarbonateions absorb the UV light, inhibit the penetration of light and thus decrease the bacterial inactivation rate[40].

Conclusion

In the present study, the resistance development in *E. coli* in wastewater against the antibiotic ciprofloxacin and the effect of UV light and UV/H_2O_2 method on *E. coli* inactivation process was studied. The antibiotic resistance of *E. coli* in the municipal wastewater of College Ghat, Howrah were 69 times resistances against ciprofloxacin. Particularly, UV/H_2O_2 method is more active for the *E. coli* inactivation in wastewater than the UV exposure. At 4 h of treatment, UV/H_2O_2 process achieved an inactivation of approximately 99% of antibiotic resistance *E. coli*; whereas UV light treatment achieved 61% inactivation in synthetic water. In real wastewater, about 86% of antibiotic resistance *E. coli* was inactivated by combined UV/H_2O_2 method. The first order UV/H_2O_2 rate constant on *E. coli* inactivation in real wastewater was found to be increased with increasing peroxide dose (0.0049 to 0.0087 min^{-1}). The UV/H_2O_2 method for the inactivation of antibiotic resistant *E. coli* in municipal wastewater is highly effective because the hydroxyl radical produced by the photolysis of hydrogen peroxide bond may inactivate enzyme, damage intracellular organs and also hampered the function of protein synthesis of the bacterial cell etc.

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