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Application of response surface methodology for the optimization of oxacillin degradation by subcritical water oxidation using H_2O_2: genotoxicity and antimicrobial activity analysis of treated samples

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ABSTRACT

In this study, degradation of oxacillin in initial solution of 80 ppm was achieved providing high yield of total organic carbon removal, using a powerful, simple and safe method. Two models of response surface method, namely Box–Behnken design (BBD) and central composite design applied to optimize experimental process and theoretical equations were proposed. The *F* and R^2 values were obtained as 76.81 and 0.9900, respectively, in the case of BBD and 187.07 and 0.99641, respectively, in the case of CCD. The highest experimental and predicted values of total organic carbon removal rates were obtained as 75.88% and 75.49%, respectively, under operating conditions such as 373 K of temperature, 35 mM of concentration of H_2O_2 and 45 min of treatment time in the case of BBD. In the case of CCD, the values were obtained as 76.42% and 76.97%, respectively, under operating conditions such as 373 K of temperature, 55 mM of concentration of H_2O_2 and 45 min of treatment time. In addition, the reliability of this work lies on genotoxic effect and antimicrobial activity analysis of treated samples. Thus, treated samples did not show any genotoxic effect, and antimicrobial activity was reduced.

Keywords: RSM; CCD; Total organic carbon removal; ANOVA; β-Lactam antibiotic; Degradation; Subcritical water; SWO

1. Introduction

Pharmaceuticals consumption for treatment of diseases faced by human, animals and plants has increasingly extended. Recently, pharmaceuticals are produced in large amounts and they are introduced into the environment [1,2]. These compounds are not completely absorbed or metabolized in the body and high percentage of them is eventually excreted [3,4]. Discharging drugs into municipal, industrial and hospital effluents cause water contamination and development of resistant bacteria [5,6].

β-Lactam antibiotics constitute a wide class of semi-synthetic penicillins that are widely used due to their antimicrobial activity against bacterial diseases for decades [7]. Antimicrobial activity of β-lactams occurs from β-lactam ring and variable side chains are responsible for the differences in their pharmacological and chemical properties [8]. Degradation of β-lactam antibiotics has gained great attention of researchers due to their excessive usage and presence in water sources [9].

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Oxacillin (OXA) is a kind of narrow-spectrum β-lactam antibiotic used for its activity against gram-positive bacteria [10]. OXA has been detected in the wastewater treatment plants and natural waters [8,11,12]. Existence of antibiotics in aquatic environment can promote resistance in natural bacterial populations and can cause ineffective treatments of several diseases [13,14]. OXA-resistant bacteria can reinfect humans and lead to serious health problems as many pathogenic bacteria caused [15]. Antibiotic resistance is considered as one of the most important health problems by researchers [16]. Therefore, efficient processes are promptly required for the degradation of OXA in water medium.

Although several conventional methods such as biological oxidation, phytoremediation, chemical coagulation/flocculation, adsorption and advanced oxidation have been applied for treatment of waters containing various hazardous contaminants, they have serious limitations and their applicability is restricted [17–20]. Water heated between 373 and 643 K and maintained under enough pressure to keep it in liquid form is called subcritical water [21]. Subcritical water oxidation (SWO) is considered as an effective method of degradation by researchers offering many advantages [22–24]. SWO provides favorable medium for the formation of hydroxyl radicals (HO•) and various oxidizing species that take major part in degradation process [25]. The effectiveness of SWO has been proven in the degradation of hazardous pollutants including antibiotics using hydrogen peroxide which is an environmentally friendly oxidizing agent [26–29]. In spite of H_2O_2 decomposing to O_2 and H_2O at room temperature, it shows synergistic effect with subcritical water resulting in an enhanced formation of active radical species and an increased degradation rate of target compounds [29]. Furthermore, SWO requires short treatment times comparing with conventional methods, and thus reduces the cost of the process. Hence this method can be used as an advantageous alternative to traditional methods in the degradation of contaminants present in aqueous media.

In the present research, the degradation of OXA was investigated using subcritical water medium and H_2O_2. Synthetically prepared OXA solution was selected as a model for wastewaters contaminated by OXA. Response surface method (RSM) was used to exhibit optimum experimental parameters (temperature, treatment time and concentration of oxidizing agent) and applicable theoretical function for predicting and determining further response [18,30].

Chemometric tools involving a series of mathematical and statistical methods that offer advantages such as minimizing the reagent consumption and laboratory work, saving experimental time and personal costs and optimizing experimental parameters have been widely applied for the optimization of analytical methods [30–32]. Thus, these methods are useful to improve the performance of a system for obtaining maximum benefit in experimental design [33]. Furthermore, approximation models for experimental data can be provided and interaction of the independent variables and major effects of the system can be easily assessed [30,34]. RSM is a collection of the several effective types of experimental design methods used for optimizing multivariable chemical process and evaluating the performance of a system and the interaction effects of independent variables [18,34]. Box–Behnken design (BBD) and central composite design (CCD) are two types of design that have been widely employed in researches among the various RSM approaches [33,35,36]. BBD and CCD were performed to evaluate the experimental factors and their interaction effects and to obtain optimum working conditions and determine approximation models in the OXA degradation.

In the second part of this study, genotoxicity of treated samples was also evaluated. Although main aim is degrading hazardous compounds to CO_2 and H_2O, many toxic and persistent by-products may originate in the degradation process. Thus, genotoxic effects of treated samples should be evaluated by genotoxicity tests (such as comet assay, micronucleus and nuclear abnormalities) [37]. Comet test has been used to evaluate both the genotoxicity and toxicity of many different chemicals, drugs and nanoparticles [38]. Comet assay which is known as single cell gel electrophoresis provides a suitable tool for quantifying level of DNA repair and DNA single and double strand breaks at the level of individual cells [39]. It is also widely accepted as a very sensitive and rapid method [40].

For the later part, antimicrobial activity analysis of treated samples was determined due to the fact that degradation products may exhibit more antimicrobial effects to bacterial strains in comparing with the stock OXA solution. Also, correlation between the degradation rates and antimicrobial activity of treated samples was assessed.

2. Materials and methods

2.1. Materials

Oxacillin sodium was obtained from Sigma-Aldrich (United States). H_2O_2 and total organic carbon (TOC) cell kits were purchased from Merck (Germany), N_2 gas was supplied by Linde gas (Turkey), ultra-pure water was prepared using Millipore Milli-Q Advantage A10. Culture medium Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum, penicillin–streptomycin solution and amphotericin B solution were purchased from Biological Industries (United States). PBS, EDTA, Triton X-100, normal melting point agarose, low melting point agarose were purchased from Sigma-Aldrich (United States). Materials used for bacterial growth media preparation and catalase (EC 1.11.1.6, CAS Registry No. 9001- 05-2, activity 10.000 U/mg protein) were purchased from Merck (Germany) and Sigma-Aldrich (United States), respectively. Sterile 96-Well Plates were obtained from Polar Chemicals (Turkey).

2.2. Experimental procedure

All degradation runs were performed in triplicate at three different temperatures (373, 423, and 473 K), three treatment times (15, 35 and 55 min), and three oxidant concentrations (15, 30 and 45 mM) with oxidizing agent H_2O_2 as demonstrated in Table 2. Pressure was fixed at 30 bar supplied by nitrogen. Degradation experiments were carried out under treatment conditions using a home-made stainless steel reactor (200 mL) with a magnetic stirrer [29]. Aqueous solutions were prepared weekly and stored at 4°C. Initial concentration of stock OXA solutions was fixed at 80 ppm by dissolving specific amounts of OXA. TOC of stock solution was calculated as 43 ppm. Experimental conditions were selected according to the fact that pharmaceutical pollutants are present in low

levels in the aquatic environment based on literature [5]. Also, pre-experiments were performed before selecting working parameters and our previous works were considered [22,29].

2.3. TOC analysis

As is well-known, TOC is a useful and applicable quality parameter for waters. TOC measurement has been widely carried out to qualify organic matter content of drinking waters and wastewaters [41]. This method is based on measuring total CO_2, which is formed through oxidation of organic matter [42].

TOC cell kit and Merck Nova 30 A Spectroquant photometer which can measure TOC removal percentages of 5–100 ppm were used for TOC analysis of fractions collected at the end of the treatment time. Experimental TOC removal rates of OXA were calculated according to equation given in our previous work [29]. Collected samples were analyzed to evaluate genotoxicity and antimicrobial activity of treated OXA solution.

2.4. Experimental design of degradation process using BBD and CCD

RSM is a practical tool used to assess optimum degradation parameters, and to ensure applicable theoretical function through carrying out limited number of experiments. Features and theoretical basic of frequently used BBD and CCD models are briefly discussed here.

BBD is based on three-level incomplete factorial design and provides calculation of the response and enables efficient estimation of the system performance at all levels within the studied range by designing the process carefully [43,44]. It is an independent, spherical and rotatable or nearly rotatable, second-order (quadratic) RSM design that consists of points located on the center and middle of cube restricted with its sphere. The number of experiments required for BBD is given by $N = 2k\ (k - 1) + C0$, where k is the number of factors and C0 is the replicate number of central points [45,46]. BBD offers an economical and effective alternative to 3k designs, as it requires less number of experiments. Although 3k designs require 27 runs in three-level optimization, BBD requires 12 plus 5 replicates in central point. BBD mostly constitutes a suitable alternative design to CCD [47]. Thus, this experimental design method has been applied in several chemical and physical processes and its popularity has increased in industrial community [48]. CCD, which has been widely used in analytical chemistry, was first introduced by Box and Wilson [49,50]. It is probably the most common statistical design used for evaluation of second-order RSM models. This method consists of a full factorial or fractional factorial designs with an additional star points and at least one point at the center of experimental region [46]. The total number of experiments required for CCD is given by $N = 2k + 2k + C0$, where k is the number of variables and C0 is the replicate number of central points [33].

In this study, two types of RSM designs, BBD and CCD were employed for optimizing the process and determining the relationship between independent variables. The efficiency of these methods was compared. The effect of three independent variables, which are crucial in SWO using H_2O_2, namely temperature (x_1) in K; concentration of oxidizing agent (x_2) in mM and treatment time (x_3) in min were investigated at three levels. TOC removal percentage (%) was assumed as the dependent variable (response, Y). The experimental design of the independent variables in both designs are shown in Table 1. Results were statistically evaluated using Design Expert 9.0.6.2 version and the optimum model equations were proposed.

The design was expressed by linear or quadratic models, in which response can be simply correlated with the independent variables. A quadratic model, which also contains the linear model, is shown in Eq. (1):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \varepsilon \quad (1)$$

In this equation, Y symbolizes the approximation response, TOC removal percentage, and x_1, x_2 and x_3 represent the coded independent variable effects, and x_1^2, x_2^2 and x_3^2 show the square effects. x_1x_2, x_1x_3 and x_2x_3 show interaction effects. β_1, β_2 and β_3 demonstrate the linear coefficients. β_{11}, β_{22} and β_{33} represent the square coefficients. β_{12}, β_{13} and β_{23} are the interaction coefficients. β_0 and ε represent the constant and the random error, respectively [29].

According to BBD and CCD models, 17 and 20 experiments were performed, respectively, in which center point of the experimental region was replicated five and six times,

Table 1
Experimental design of the independent variables used in RSM

Factors	Independent variables	Coded levels				
		–2	–1	0	1	2
BBD						
x_1	Temperature (K)	–	373	423	473	–
x_2	Concentration of oxidizing agent (mM)	–	15	30	45	–
x_3	Treatment time (min)	–	15	35	55	–
CCD						
x_1	Temperature (K)	338.9	373	423	473	507.1
x_2	Concentration of oxidizing agent (mM)	4.8	15	30	45	55.2
x_3	Treatment time (min)	1.4	15	35	55	68.6

respectively. Experiments were performed in randomized order as is common in many design procedures. Analysis of variance (ANOVA) was performed to determine the proposed models' accuracies and statistically significant differences between independent variables. Quadratic polynomial models were evaluated and their properties expressed by the coefficients of determinations (R^2, R^2_{adj}), Fisher's '*F*' test, and *P* value.

2.5. Genotoxicity analysis

For this study, two healthy male donors (volunteers) who are 28 and 29 years of age and had no history of malignant diseases have been chosen. Comet assay was performed with lymphocytes of two donors according to Tice et al. [51]. Heparinized blood samples were obtained by venous puncture for comet assay. Lymphocytes were isolated by centrifugation with Histopaque-1077. All cultures were set up by adding 0.5 mL of lymphocyte suspension in 4.5 mL of solution containing RPMI, fetal bovine serum, L-glutamine, phytohemagglutinin, penicillin and streptomycin. Cells were incubated at 37°C for 24 h with stock OXA solution and treated samples of OXA degradation experiments. After incubation treatment, first, 0.1 mL of cell suspension was mixed with 0.2 mL of low melting point agarose at 37°C. Then, this mix was placed on a slide pre-coated with normal melting point agarose and the cell suspension was immediately covered with a cover-glass and these slides were kept at 4°C until further analysis. Following the process, slides were exposed to lysis solution and denatured in alkaline buffer. Finally, these slides were washed with neutralization buffer and ethanol. The process was performed in dark to prevent DNA damage. These slides were air-dried and stained with ethidium bromide and analyzed by the Olympus BX40 fluorescence microscope. Statistical analysis was performed using SPSS software package. Differences between mean values were compared using one-way ANOVA. The Bonferroni test was used at Post hoc analysis. $P < 0.05$ was considered as level of significance.

2.6. Antimicrobial activity analysis

2.6.1. Evaluation of residual antimicrobial activity of treated OXA samples

The residual antimicrobial activity against gram positive (*Bacillus subtilis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*) and gram negative (*Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*) bacteria was assessed by the agar well diffusion method [10]. One loop colonies of bacterial strains from agar plates were inoculated into 10 mL Mueller-Hinton broth including 2 g/L meat infusion, 17.5 g/L casein hydrolysate and 1.5 g/L starch and incubated at 37°C for 24 h. Population density of culture was calculated by McFarland standard and 100 μL bacterial suspension containing 1.0×10^6 CFU/mL cell was spread on to Mueller-Hinton Agar. Degradation products (150 μL) were transferred to holes into Mueller-Hinton Agar. The plates were incubated at 37°C for 48 h. Stock OXA solution was used as control. Residual hydrogen peroxide in degraded solutions was removed through catalase treatment before antimicrobial assay.

2.6.2. The effect of treated OXA samples on bacterial growth

The effect of treated samples on growth of *B. subtilis*, *S. pneumoniae*, *S. aureus*, *E. faecalis*, *K. pneumoniae* and *P. vulgaris* which were sensitive to OXA was determined. Sterile 96-well microtiter plate was used for assay. 300 μL of Luria Bertani broth (LB) (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) was prepared with distilled water and transferred into first well. LBs prepared with stock OXA solution and treated samples which of TOC removal rates obtained as 26.07% (S1), 48.00% (S2), 64.68% (S3) and 65.91% (S4) were added to 2–6 well, respectively. Then, 6 μL of bacterial culture dilution containing approximately 1×10^6 CFU/mL was added to each well (1–6). The increase in optical density at 600 nm was monitored by Multiscan Gomicroplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) during 24 h incubation periodically. *Bacillus subtilis* was incubated at 150 rpm and 30°C while incubation was performed at 150 rpm and 37°C for *S. pneumoniae*, *S. aureus*, *E. faecalis*, *K. pneumoniae* and *P. vulgaris*. All tests were performed in triplicate.

3. Results and discussion

3.1. Optimization of degradation of OXA using BBD and CCD

BBD and CCD were employed to identify effects of system variables and to obtain the relationship between the variables and the response in degradation process of OXA. Predicted and experimental values of TOC removal rates and actual/coded values of independent variables obtained from BBD and CCD are shown in Table 2. Table 2 demonstrates that the highest and lowest TOC removal rates were obtained at runs 12 and 7 as 75.88% and 38.14%, respectively, in the case of BBD and at the runs 2 and 17 as 17.86% and 76.42%, respectively, in the case of CCD.

ANOVA results of quadratic models for the TOC are tabulated in Table 3. Statistical analysis verified that quadratic models were significant for describing the effect of variables on the TOC removal rates. The model's *F* values were obtained as 76.81 and 187.07 in terms of BBD and CCD, respectively, which imply that the models are significant. There are only a 0.01% chances that *F* values of this order could occur due to noise in each cases. *P* value less than 0.05 indicates that the model terms are significant. Thus, x_1, x_2, x_3, x_1x_3, x_1^2 and x_3^2 are significant model terms in the case of BBD; x_2, x_3, x_1x_2, x_1x_3, x_2x_3, x_2^2 and x_3^2 are significant model terms in the case of CCD. The results indicated that temperature, x_3, was the most favorable parameter of the degradation process that possessed the highest *F* value in both case of BBD and CCD. Moreover, lack of fit values were found to be insignificant whom *p* values were higher than 0.05. CCD was found to be better than BBD in describing the effectiveness of model and all model terms except x_1. So that, small *p* value and high *F* value specified stronger effect on the response variable for any terms of the model [35].

As is shown in Table 4, the square root of residual mean square values that are called standard deviation were obtained as 1.57 and 1.68 for BBD and CCD models, respectively, where low values are desired [52]. Predicted residual sum of squares, PRESS, is a measure of how well the model fits each point in the design [53]. PRESS value of BBD model is better than that of CCD, where small value is known as

Table 2
Predicted and experimental values of TOC removal rates and actual/coded values of independent variables obtained by BBD and CCD

Run	x_1: T (K)	x_2: C (mM)	x_3: t (min)	Y_1, Y_2: TOC removal, %	
				Exp.	Pre.
BBD					
1	423 (0)	35 (0)	30 (0)	66.78	67.60
2	473 (+1)	35 (0)	15 (–1)	49.57	49.96
3	473 (+1)	35 (0)	45 (+1)	55.63	54.66
4	423 (0)	55 (+1)	45 (+1)	74.66	74.38
5	373 (–1)	55(+1)	30 (0)	71.61	72.27
6	423 (0)	35 (0)	30 (0)	67.38	67.61
7	373 (–1)	35 (0)	15 (–1)	38.14	39.11
8	473 (+1)	15 (–1)	30 (0)	59.13	58.47
9	423 (0)	15 (–1)	45 (–1)	66.29	67.92
10	423 (0)	35 (0)	30 (0)	66.21	67.61
11	373 (–1)	15 (–1)	30 (0)	64.15	62.91
12	373 (–1)	35 (0)	45 (+1)	75.88	75.49
13	423 (0)	55 (+1)	15 (–1)	57.82	56.19
14	423 (0)	35 (0)	30 (0)	68.69	67.61
15	473 (+1)	55 (+1)	30 (0)	65.49	66.73
16	423 (0)	35 (0)	30 (0)	68.97	67.61
17	423 (0)	15 (–1)	15 (–1)	44.74	45.02
CCD					
1	473 (+1)	55 (+1)	15 (–1)	48.27	49.19
2	373 (–1)	55 (+1)	45 (+1)	76.42	76.97
3	423 (0)	35 (0)	30 (0)	66.98	67.44
4	373 (–1)	55 (+1)	15 (–1)	48.06	47.95
5	473 (+1)	15 (–1)	45 (+1)	64.89	63.70
6	423 (0)	35 (0)	30 (0)	67.78	67.44
7	423 (0)	68.6 (+2)	30 (0)	53.59	52.98
8	423 (0)	35 (0)	4.8 (–2)	26.07	26.00
9	423 (0)	35 (0)	55.2 (+2)	66.73	68.64
10	423 (0)	35 (0)	30 (0)	68.22	67.44
11	373 (–1)	15 (–1)	45 (+1)	67.35	65.13
12	473 (+1)	15 (–1)	15 (–1)	43.86	42.02
13	507.1 (+2)	35 (0)	30 (0)	64.68	66.14
14	423 (0)	35 (0)	30 (0)	66.19	67.44
15	473 (+1)	55 (+1)	45 (+1)	54.12	52.73
16	423 (0)	1.4 (–2)	30 (0)	34.55	36.99
17	373 (–1)	15 (–1)	15 (–1)	17.86	17.96
18	338.9 (–2)	35 (0)	30 (0)	65.91	66.29
19	423 (0)	35 (0)	30 (0)	68.23	67.44
20	423 (0)	35 (0)	30 (0)	67.56	67.44

better. The coefficient of determination, R^2, values were obtained as 0.9900 and 0.9941, respectively, for BBD and CCD, which indicate relationship between the parameters. R^2 values are described as a value between 0 and 1 where high values are desired [53]. Thus, CCD model was proved to be more significant over R^2 values when it is compared with BBD model. Adjusted R^2 is a measure of the amount of variation around the mean presented by the model and

Table 3
ANOVA results of quadratic model for TOC removal rates obtained by BBD and CCD

BBD			Source	CCD		
Mean square	*F* value	*P*- value prob > *F*		Mean square	*F* value	*P*-value prob > *F*
190.11	76.81	<0.0001	Model	528.45	187.07	<0.0001
49.8	20.12	0.0028	x_1	0.029	0.0099	0.9226
155.50	62.82	<0.0001	x_2	308.73	109.28	<0.0001
844.40	341.15	<0.0001	x_3	2,194.34	776.77	<0.0001
0.30	0.12	0.7369	x_1x_2	260.27	92.13	<0.0001
250.91	101.37	<0.0001	x_1x_3	324.74	114.96	<0.0001
5.55	2.24	0.1781	x_2x_3	164.80	58.34	<0.0001
77.55	31.33	0.0008	x_1^2	2.71	0.96	0.3501
13.35	5.39	0.0532	x_2^2	908.11	321.46	<0.0001
304.87	123.17	<0.0001	x_3^2	729.41	258.21	<0.0001
2.48	–	–	Residual	2.82	–	–
3.87	2.71	0.1801	Lack of fit	5.03	8.06	0.0195
1.43	1.43	–	Pure error	0.62	–	–

Table 4
Regression coefficient of the models obtained by BBD and CCD

Regression coefficients	BBD	CCD
Standard deviation	1.57	1.68
PRESS	194.67	201.93
R^2	0.9900	0.9941
Adjusted R^2	0.9771	0.9888
Predicted R^2	0.8874	0.9578
Adequate precision	30.157	49.653

predicted R^2 is a measure of the amount of variation in new data explained by the employed model [52]. The predicted R^2 of 0.8874 is in a reasonable agreement with the adjusted R^2 of 0.9771 in the case of BBD. The predicted R^2 of 0.9578 is in reasonable agreement with the adjusted R^2 of 0.9888 in the case of CCD. The adequate precision values, which measure the signal-to-noise ratio, were found to be 30.157 and 49.653 for BBD and CCD, respectively. Obtained adequate precision values were greater than 4. Thus, these values indicate that models can be used to navigate the design space [29].

According to the BBD and CCD results, the predicted second-order polynomial (quadratic) models were obtained using the Design-Expert software that are given in Eqs. (2) and (3), respectively [52].

$$Y_1 = +67.61 - 2.49x_1 + 4.41x_2 + 10.27x_3 - 0.28x_1x_2 - 7.92x_1x_3 - 1.18x_2x_3 - 4.29x_1^2 + 1.78x_2^2 - 8.51x_3^2 \quad (2)$$

$$Y_2 = +67.44 - 0.045x_1 + 4.75x_2 + 12.68x_3 - 5.70x_1x_2 - 6.37x_1x_3 - 4.54x_2x_3 - 0.43x_1^2 - 7.94x_2^2 - 7.11x_3^2 \quad (3)$$

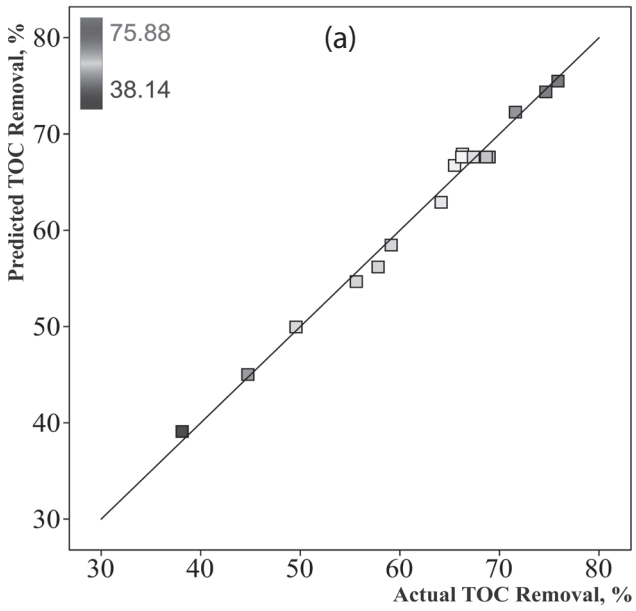
These equations in terms of coded factors can be employed to make predictions about the degradation percentage for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients [52]. It can be observed from Eqs. (2) and (3) that the sequence of efficiency of factors for predicted models in degradation process is as: $x_3 > x_2 > x_{22} > x_1x_2 > x_2x_3 > x_1 > x_{12} > x_1x_3 > x_{32}$ and $x_3 > x_2 > x_1 > x_{12} > x_2x_3 > x_1x_2 > x_1x_3 > x_{32} > x_{22}$ in the case of BBD and CCD, respectively. Y_1 and Y_2 correspond to the response of degradation of OXA as TOC removal percentage, respectively, performing BBD and CCD.

Relations between predicted and experimental values of TOC removal rates that are presented in Figs. 1(a) and (b) obtained by model equations (Eqs. (2) and (3)) using BBD and CCD, respectively. Both figures show that the points belong to the predicted values match with experimental points, verifying a good fit of models. In addition, this accordance is supported by the R^2, adjusted R^2 and predicted R^2 values shown above in Table 4.

Figs. 2(a) and (b) are demonstrated to facilitate the evaluation of data composed of three independent variables with highest and lowest value of each at the middle of each edge and each corner in the case of BBD and CCD, respectively. Details about the prediction and the observation can be displayed using Figs. 2(a) and 2(b). In addition, these figures can be used to assist to design further experimental setup in preferred conditions.

Figs. 3(a)–(c) and Figs. 4(a)–(c) demonstrate the three-dimension plots displaying the effect and interaction of independent variables of temperature, oxidant concentration and treatment time on TOC removal percentage of OXA using BBD and CCD, respectively.

Fig. 3(a) shows the interaction between temperature and treatment time and effect of them on the TOC removal rates of OXA at a fixed H_2O_2 concentration of 45 mM obtained by BBD. It is clearly shown that long treatment time has a positive effect at medium and high temperatures values, while high temperature inversely has a negative effect on TOC removal rates at all treatment times. Short treatment times were found to be effective for significantly high TOC removal rates at lower temperature. Specific time which is thought to be responsible for enhanced TOC removal rates may have been required for the formation of more free radicals. While



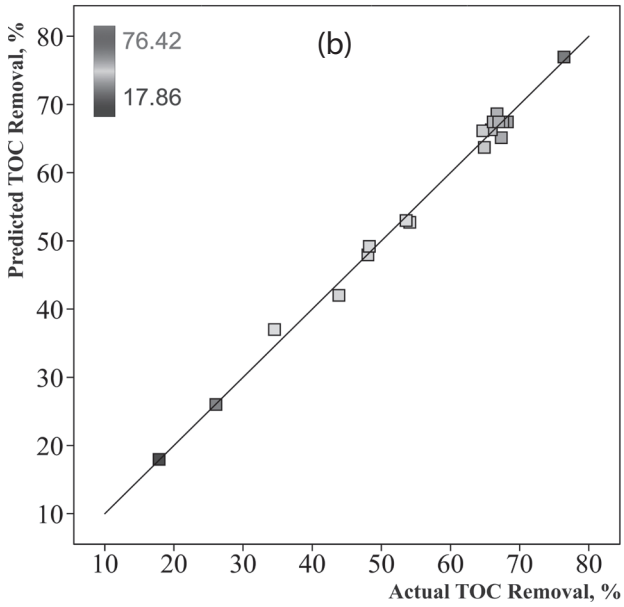
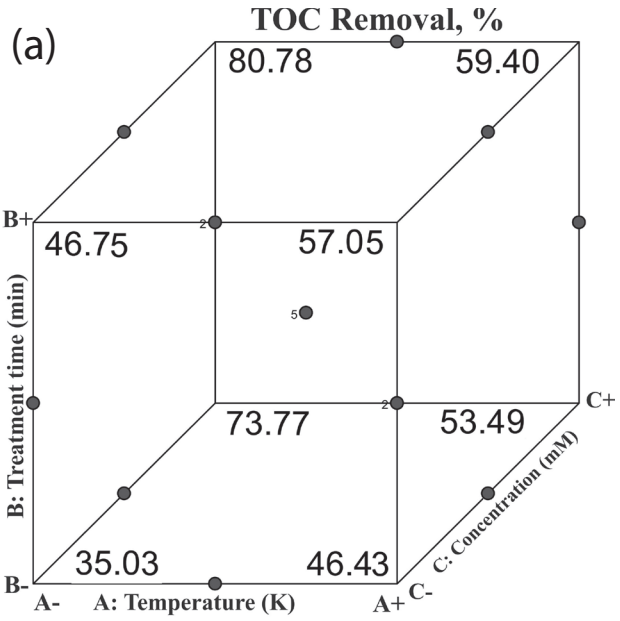


Fig. 1. (a) and (b) Relation between the actual and predicted values of TOC removal using BBD and CCD.



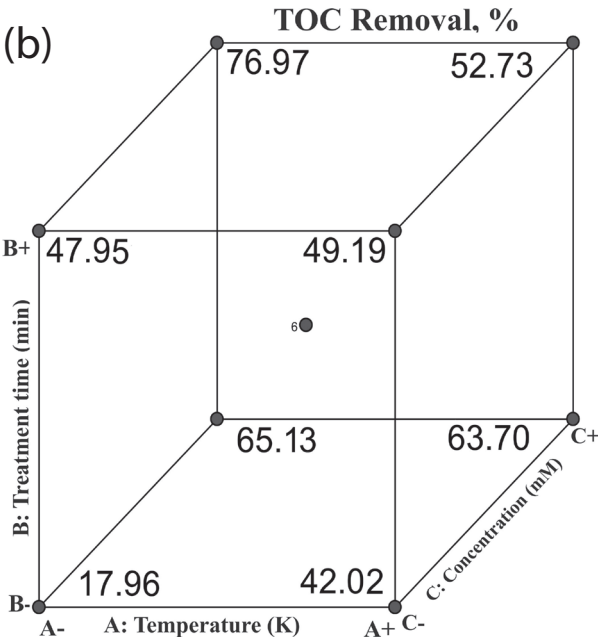


Fig. 2. (a) Cube plot that shows the predicted values from the coded model for the combination of the –1 and +1 levels of any three factors for response, TOC removal percentage, using BBD. (b) Cube plot that shows the predicted values from the coded model for the combination of the –1 and +1 levels of any three factors for response, TOC removal percentage, using CCD.

the treatment time enhanced the rates at 373 K significantly, same enhancement was not observed at 473 K. Reasonably high rates were obtained under the combined effect of temperature and treatment time of 373 K and 55 min. Giraldo-Aguirre et al. [10] achieved 100% OXA removal in 120 min of treatment time while they indicated that 480 min of treatment time was required for 100% mineralization. Considering their results, one can say that 55 min of treatment time is more preferable.

Fig. 4(a) shows the interaction between temperature and treatment time and effect of them on the TOC removal rates of OXA at a fixed H_2O_2 concentration of 45 mM obtained by CCD. Comparing Figs. 3(a) and 4(a), it can be concluded that medium values of treatment time are more effective than that of high values of treatment time at all temperature in the case of CCD. This difference can be attributed to the characteristics of CCD model that required more experiments in wider schedule.

The integrated effect of temperature and concentration of oxidizing agent, H_2O_2, was demonstrated in three-dimensional plots in Figs. 3(b) and 4(b) obtained by BBD and CCD, respectively. It is clearly seen from both figures that

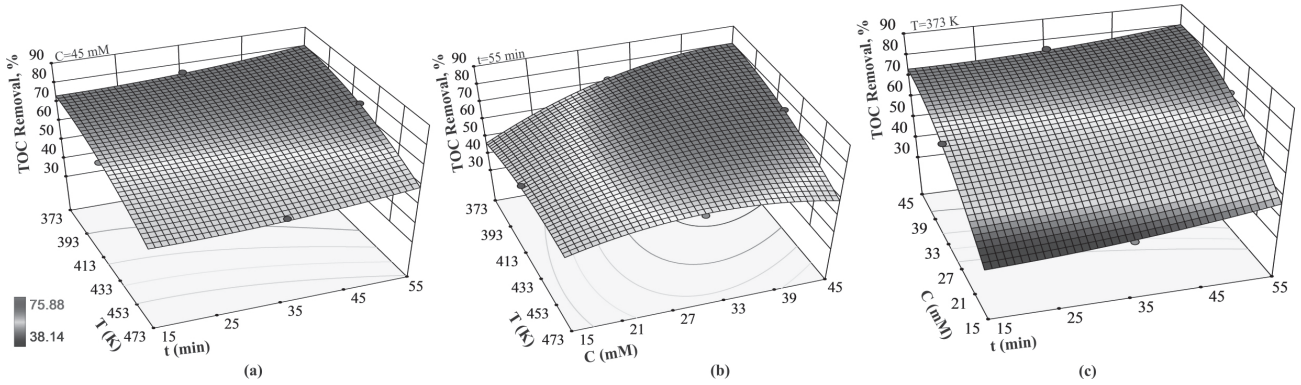


Fig. 3. Three-dimensional surface plot of TOC removal of OXA using BBD, (a) vs. temperature (*T*) and treatment time (*t*) at fixed *C* = 45 mM, (b) vs. temperature (*T*) and concentration (*C*) at fixed *t* = 55 min, (c) vs. concentration (*C*) and treatment time (*t*) at fixed *T* = 373 K.

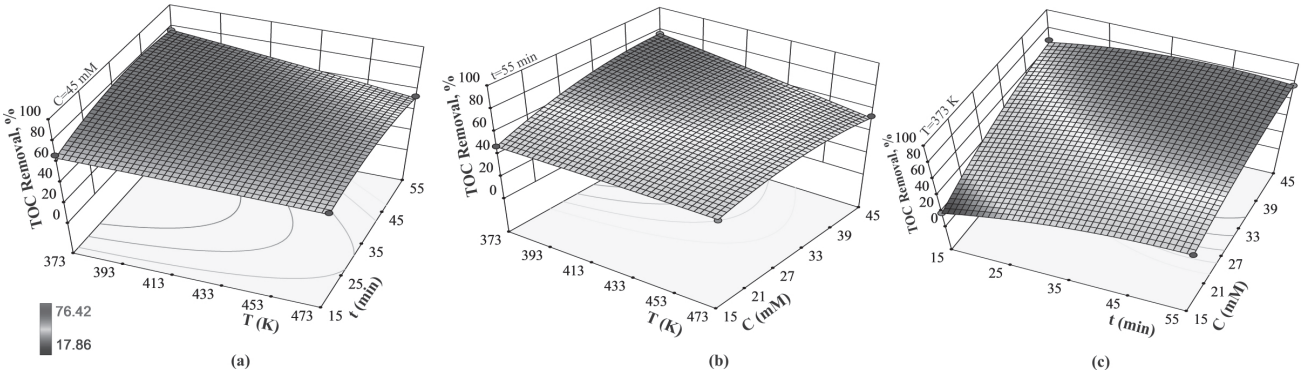


Fig. 4. Three-dimensional surface plot of TOC removal of OXA using CCD, (a) vs. temperature (*T*) and treatment time (*t*) at fixed *C* = 45 mM, (b) vs. temperature (*T*) and concentration (*C*) at fixed *t* = 55 min, (c) vs. treatment time (*t*) and concentration (*C*) at fixed *T* = 373 K.

TOC removal rates remained low at low values of concentration and temperature. The efficiency of TOC removal considerably increased in the high concentration of H_2O_2 at low and medium value of temperature according to BBD model and at almost all value of temperature, according to CCD model. Figs. 3(b) and 4(b) proved that elevated TOC removal rates are highly dependent on concentration of H_2O_2 providing an increase in the concentration of •OH radicals which react with the worked compound and therefore enhance the efficiency of process [19]. Relatively high TOC removal rate was obtained at moderate temperature levels at constant H_2O_2 concentration at or above 30 mM. High temperature values were found to reduce TOC removal rates, regardless of H_2O_2 concentration, attributed to the possible degradation of the H_2O_2 and formation of chain reaction between •OH radicals and its derivatives resulting in quenching of them which pointed out in our previous work [29].

Considering Figs. 3(c) and 4(c), it can be concluded that higher TOC removal rates obtained at high concentration of H_2O_2 and treatment time due to interactive effect of both variables at a constant temperature of 373 K. Also, high treatment time levels provide elevated TOC removal rates at medium and high levels of oxidant concentration while effect of treatment time remains limited at low level of concentration of H_2O_2 at a constant temperature of 373 K according to Fig. 3(c). In addition, increasing treatment time from 15 to 45 min, increases TOC removal rates from 38.14% to 75.88% using 35 mM of H_2O_2 at 373 K in the case of BBD. Increasing concentration of H_2O_2 from 15 to 55 mM increases TOC removal rates from 17.86 to 48.06 and increases the rates from 67.35 to 76.42 at 15 and 45 min, respectively, at 373 K in the case of CCD. Thus, concentration of H_2O_2 is more effective at low treatment time than that of higher treatment time on the efficiency of degradation according to Fig. 4(c). These findings can be attributed to the fact that specific time is required for formation of adequate •OH radical and its powerful derivatives and this effect remains limited at higher treatment time. These results were found to be compatible with our previous finding for degradation of cloxacillin (77% of TOC removal was obtained) which belongs to β-lactam antibiotics [29].

3.2. Antimicrobial activity analysis of treated samples

Disposal or at least reduction of antimicrobial activity of treated samples is as essential as elevation of TOC removal rate for successful antibiotic degradation process. For this purpose, retained antibacterial activity of four samples which have different TOC removal rates (S1, S2, S3 and S4) was evaluated by agar well diffusion method. In general, β-lactams like OXA affect gram positives more

than gram negatives. However, gram positives (*B. subtilis*, *S. pneumoniae*, *S. aureus* and *E. faecalis*) were used in our study as well as gram negatives (*E. coli*, *A. baumannii*, *K. pneumoniae*, *P. vulgaris* and *P. aeruginosa*) the reason being some degradation by-products formed during the process might influence gram negatives. Negative control (distilled water) did not influence the growth of all bacterial strains on agar surface. Inhibition zones around wells filled with untreated OXA solution were measured as 23, 60, 33, 35, 81 and 21 mm for *B. subtilis*, *E. faecalis*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, *S. aureus* and *S. pneumoniae*, respectively. Inhibition zone was not observed for stock OXA solution using *A. baumannii*, *E. coli* and *P. aeruginosa*. Also, antimicrobial effect was not observed for treated OXA samples using all tested bacterial strains (Fig. 5).

The profile of the bacterial population concentration (as OD_{600}) in LB medium prepared with distilled water (LB-1), stock OXA solution (LB-OXA) and treated OXA samples (LB-S1, LB-S2, LB-S3 and LB-S4) is shown in Fig. 6. Standard growth profiles were obtained by measuring absorbance (OD_{600}) of LB-1 at regular intervals during incubation of bacterial strains. Effect of stock and treated samples on bacterial growth was assessed via comparing standard growth profile. Stock OXA and treated OXA solutions did not have noticeable effect on *A. baumannii*, *E. coli* and *P. aeruginosa* (data not shown). While growth of *E. faecalis*, *S. aureus* and *S. pneumoniae* in LB-OXA was significantly inhibited, LB-OXA moderately affected on growth of *P. vulgaris*, *B. subtilis* and *K. pneumoniae*. Significant decrease in growth level was recorded when *E. faecalis*, *S. aureus* and *S. pneumoniae* incubated in LB-S1. In contrast, growth was slightly inhibited in LB-S2, LB-S3 and LB-S4 for all bacterial strains. Also, total bacterial population concentration in LB-S3 and LB-S4 was found higher than LB-S2. These results showed that the reduction in inhibitory effect of treated OXA solutions on growth of OXA-susceptible bacterial strains are related to the TOC removal percentages.

In previous studies, quality of the treatment methods for OXA elimination has been confirmed by reduction in antimicrobial activity [2,10,12]. Serna-Galvis et al. [2] reported that the antimicrobial activity against *S. aureus* was reduced by treatment of OXA solution using photo-Fenton, TiO_2-photocatalysis and electrochemical processes. While, they obtained significant reduction in antimicrobial activity scarcely under conditions which 80%–100% of OXA

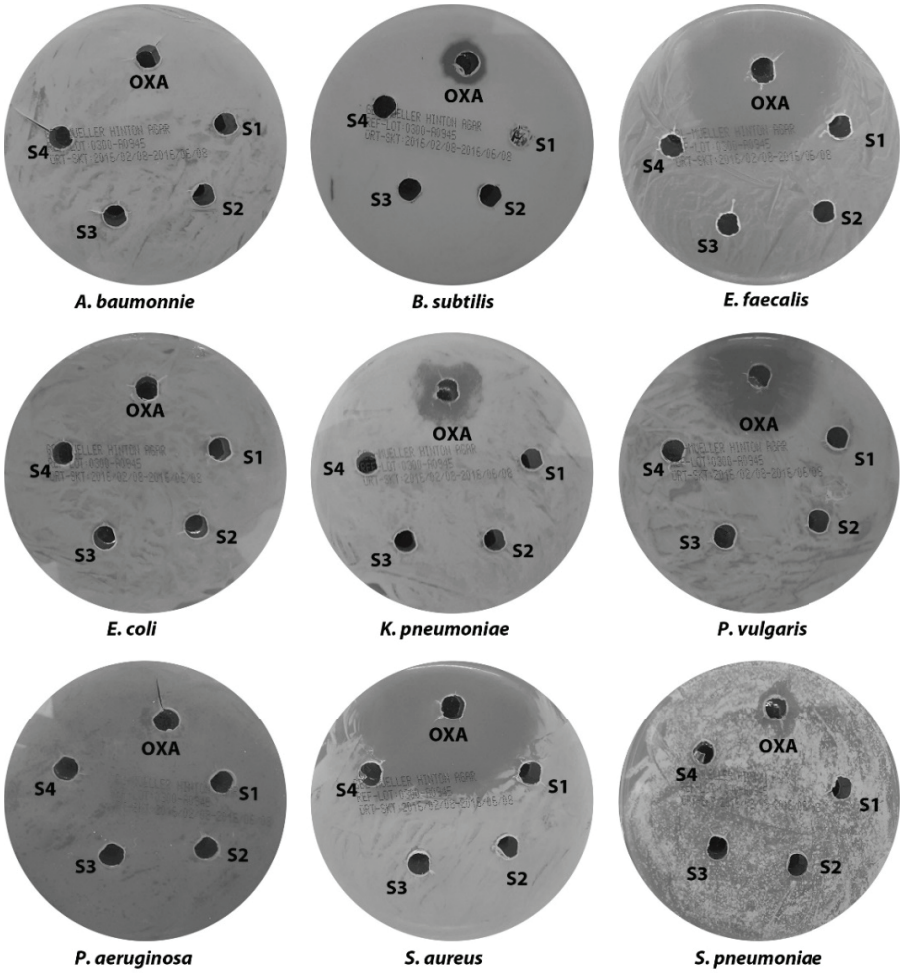


Fig. 5. Inhibition zones on Mueller-Hinton Agar with gram-positive (*B. subtilis*, *S. pneumoniae*, *S. aureus* and *E. faecalis*) and gram-negative bacterial strains (*E. coli*, *A. baumannii*, *K. pneumoniae*, *P. vulgaris* and *P. aeruginosa*) obtained by stock OXA solution and treated samples (S1, S2, S3 and S4).

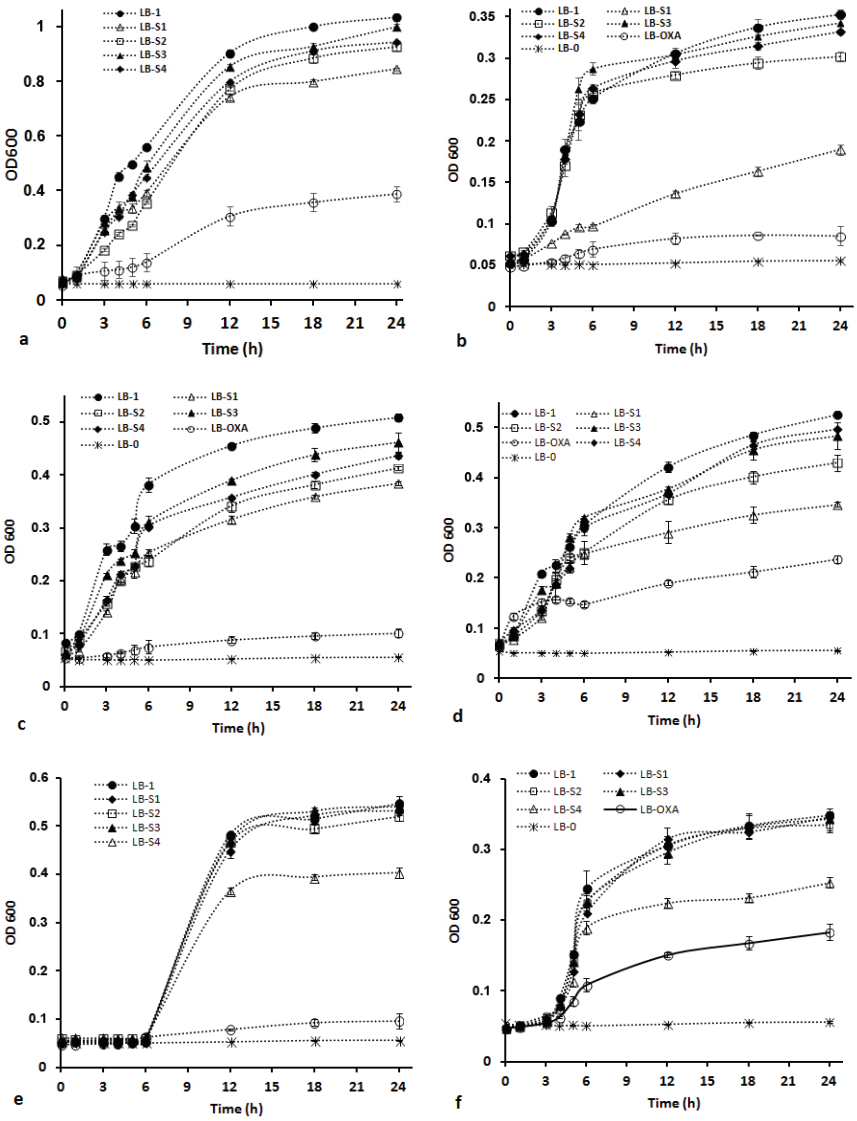


Fig. 6. The population growth profile (as OD_{600}) of the *P. vulgaris* (a), *E. faecalis* (b), *S. pneumoniae* (c), *B. subtilis* (d), *S. aureus* (e) and *K. pneumoniae* (f) in LB-1, LB-OXA and LB-S1, LB-S2, LB-S3, LB-S4, LB-0 (negative control).

elimination observed, we achieved at least 50% of reduction in antimicrobial activity of treated OXA samples for each tested samples.

3.3. Evaluation of the genotoxic effect of the OXA and the treated samples

Genotoxic effects of the stock OXA solution and the treated samples were evaluated in human lymphocytes culture. Lymphocyte cultures were established from heparinized blood samples obtained from two young, healthy male donors. Table 5 shows the results of DNA damage (comet) in donors. The comet assay is widely used to estimate genotoxic effect of several chemicals such as pesticide, nanoparticle and antibiotics [54–56]. In this study, hydrogen peroxide (100 μM) was used as a positive control. Five experiment groups containing stock OXA solution and four treated samples (S1, S2, S3 and S4) were analyzed. Significant increase was not observed in DNA damage for four tested samples in comparing to the negative control. Moreover, obtained results for treated samples were found to be very similar to that of the stock OXA solution. Similarly, increasing degradation rate did not cause a significant increase in the percentage of DNA damage. But, the positive control, hydrogen peroxide, significantly induced the DNA damage in comparison with all treated, stock OXA solution and negative samples.

Recently, many studies related to genotoxic effect of main compounds and treated samples have been reported [60–62]. Thus, genotoxic effect of stock OXA solution and treated samples were analyzed using comet assay. Reasonably, significant increase was found only in the positive control by means of genetic damage index (GDI) according to Table 5 ($P < 0.001$; 2.01 ± 0.32). Additionally, significant differences were not found between the values of treated and negative control groups. However, the

Table 5
Analysis of DNA damage as measured by comet assay in lymphocytes exposed to oxacillin and treated samples

Treatment groups	Proportion of damaged nuclei (%)[a]					Proportion of damaged cells, %[b]	GDI[c]
	Type 0	Type I	Type II	Type III	Type IV		
Negative control	74.25	15.00	4.75	3.25	2.75	10.75 ± 0.95	0.45 ± 0.03
Positive control	12.50	31.75	14.25	12.00	29.50	55.75 ± 7.63	2.01 ± 0.32*
Sample 1	75.75	12.25	3.00	3.75	5.25	12.00 ± 1.63	0.50 ± 0.04
Sample 2	78.25	11.50	4.25	2.75	3.25	10.25 ± 1.89	0.41 ± 0.05
Sample 3	78.25	13.50	2.50	2.25	3.50	8.25 ± 1.70	0.39 ± 0.02
Sample 4	75.25	14.75	5.75	2.25	2.00	10.00 ± 2.16	0.41 ± 0.03
Stock OXA	71.00	16.25	5.50	3.25	4.00	12.75 ± 1.25	0.53 ± 0.04

[a]0–IV indicate proportion of DNA damage [57].
[b]Proportion of damaged cells = Type II + III + IV [58].
[c]Genetic damage index (GDI) = (Type I + 2 Type II + 3 Type III + 4 Type IV)/(Type 0 + I + II + III + IV) [59].
*$P < 0.001$.
Type 0, undamaged; Type I, low damage; Type II, medium damage; Type III, high damage; Type IV, complete damage.

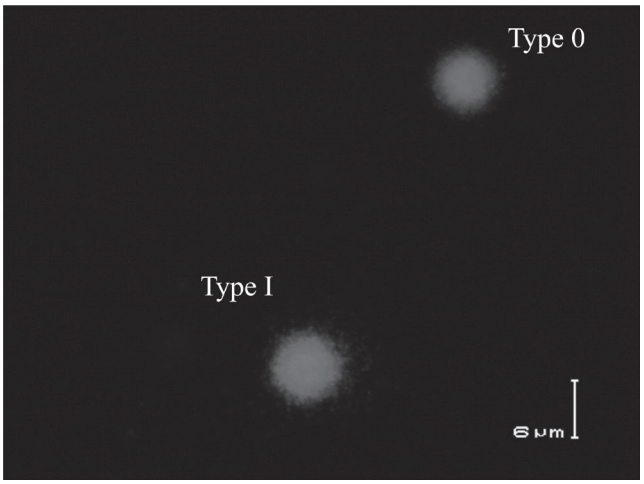


Fig. 7. DNA damage in peripheral blood lymphocytes treated with the stock OXA in vitro obtained from comet assay.

level of damaged cells was observed similar to S1 (12.00 ± 1.63) and stock OXA (12.75 ± 1.25) (Fig. 7). The lowest level of damaged cells was found in the S3 (8.25 ± 1.70). Consequently, evaluation and degradation of antibiotics and its residuals in aquatic ecosystem are major necessities. However, biotransformation of antibiotics is limited and complete degradation of the antibiotics does not occur [37]. Moreover, the degradation products may have genotoxic potential and the genotoxic potential depends not only on the degradation products but also on the characteristics and types of the pollutants, as well as the type of the degradation process [63].

4. Conclusions

The degradation of OXA was achieved in high TOC removal percentages using eco-friendly SWO method and an effective oxidizing agent, H_2O_2. The effects of temperature, concentration and treatment time on the degradation of OXA and interactions between each parameter were fully evaluated under various conditions. Best fit values of variables were 373 K, 55 mM and 45 min for temperature, concentration and treatment time where 76.42% TOC removal obtained in the case of CCD. Treatment time was found to be the major contributing variable to the TOC removal of OXA. Optimization of the degradation process and evaluation of results were performed, and approximation models for TOC removal of OXA were proposed using statistical design method, RSM. The results show that the effect and predictability of BBD and CCD designs depend on the case of the optimization of the degradation process. ANOVA results demonstrate the practicality of the models and accordance of the model terms. In addition, based on the importance of evaluating the effect of final degradation products, genotoxic effect and antimicrobial activity of treated samples were enlightened. Consequently, antimicrobial activity of treated samples reduced in contrast to stock OXA solution. Thus, minimization of potential impact of OXA on growth of bacteria is exhibited. Nevertheless, the results indicated that proposed work could be used to completely degrade OXA in the artificially contaminated water that any genotoxic effect of degradation products of OXA not seen.

Acknowledgments

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