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INVESTIGATION OF GROWTH KINETICS OF SOME PATHOGENIC BACTERIA IN AGROCHEMICAL WASTEWATER TREATED BY SUBCRITICAL WATER OXIDATION METHOD

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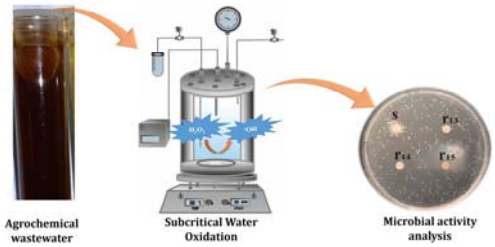
This work aimed to evaluate the potential bacterial growth kinetic of degradation by-products of the industrial agrochemical production plant wastewater that may occur after the treatment. Specific growth rate (SGR) and times of the lag-phase (t_{lag}) of *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were investigated in the presence of raw and Subcritical water oxidation method applied (treated) wastewater samples. No growth inhibition was found in both stock (raw) and Subcritical water oxidation method applied samples using disc diffusion method. According to the results of growth kinetics, especially the organic matter remained in run 14 was insufficient for the growth of the *K. pneumoniae* among the treated samples. The lowest SGR was in *K. pneumonia* with run 14 and tlag value was >18. The SGRs were presented as: r14>raw>r15>r13 for *E. coli*; r14>r15>s>r13>raw for *P. aeruginosa*, raw>r15>r13>r14 for *K. pneumoniae* at the end of the 24 h incubation ($p \leq 0.05$). The proliferation of *K. pneumoniae* can be prevented due to the reduction of organic load by processing wastewater using SWO.



INTRODUCTION

Water scarcity is a big problem today and this problem will grow even more in the future.[1,2] However, despite these clear facts, numerous chemical compounds that threaten human health are discharged into the water sources and these harmful compounds are taken in the human body through the water cycle.[2,3]

High quantities of agrochemicals are produced worldwide and wastewaters of the production plants were discharged into the clean water sources both during the production and consumption processes.[2] As a result, agrochemical wastewater is a growing problem, as it pollutes nature and causes many diseases beyond threatening the life of living organisms.[2,4] Although many chemical and biological applications have been applied to overcome this problem, satisfying purification levels have not been achieved due to the degradation-resistant nature of the pollutant, the ineffectiveness of the applied method, etc. Fortunately, several water samples containing pharmaceuticals, agrochemicals and other chemicals have been effectively decontaminated using eco-friendly subcritical oxidation method (SWO).[2, 5–8] Water is defined as subcritical water when heated at temperatures between boiling point

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and supercritical point and pressurized enough to keep it liquid in the applied temperatures.[3,9] Subcritical water oxidation targets the degrade the compound of interest, generally in the presence of oxidants such as oxygen, potassium persulfate or hydrogen peroxide, in the subcritical water medium.[10] Hydrogen peroxide is one step ahead of other oxidants since it does not leave any residue after application, thus being environmentally friendly and providing striking degradation efficiency.[2,3,7]

Besides, it is important to evaluate the microbial activities of the treated sample obtained after the treatment process, as well as the need for effective methods for removing the harmful chemical compounds that threaten human health by contaminating water resources. While both TOC and color removal of polluted water with a high TOC and color values can be removed at high rates, it is also necessary to determine whether the treated sample shows microbial activity due to the by-products likely to be formed in the intermediate steps. Thus, the reliability of the treatment process can be revealed in many respects. Oxacillin, a β-lactam antibiotic, containing synthetically prepared water was removed with 76.42 % using SWO method. Moreover, the reliability of the above-mentioned process was provided by antimicrobial activity analysis.[5]

Investigating the inhibitory or stimulating effect of wastewater against pathogenic bacteria is very important for the sustainability of the ecological balance. Especially, *Escherichia coli* (ATCC 25922) (*E. coli*), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (*K. pneumoniae*), which are the markers of the contamination of the wastewater mostly release into the water by humans, agricultural practices or industrial activities and cause contamination.[11,12] These bacteria are used as indicators in the determination of fecal contamination and the quality standard of drinking water is determined by whether it contains these microorganisms.[12–14] *E. coli*, *K. pneumoniae* and *P.aeruginosa* were isolated from contaminated water,[15,16] hospital wastewater,[17,18] and oil-containing wastewater[19] and petrochemical wastewater,[20] respectively. Some of these are antibiotic-resistant strains and cause major health problems when mixed with drinking water.[18,21] Nosocomial infections for *E. coli*,[22] pneumonia and urinary tract infections (UTIs) for *K. pneumoniae*,[23] and cystic fibrosis for *P.aeruginosa*[24] are the main ones.

In this paper, *E. coli*, *P. aeruginosa* and *K. pneumoniae* have been used as the standards for assessing contamination of drinking waters. In the previous study, 59.45% of TOC and 97.92% of color removal were achieved in the SWO process of the agrochemicals production plant wastewater with stock TOC value of 21000 ppm and intense dark color using hydrogen peroxide.[2] Many hazardous compounds belong to the pesticide, fungicide, insecticide, plant protection agent, xenobiotic, etc. have been reported to be found in the wastewater. Therefore, the microbial activities of the intermediate products that were likely to occur during the SWO of the treated water samples obtained at the end of the process were analysed. For this purpose, the microbial activity potentials of treated and raw samples against the indicators and whether they promote bacterial growth at the end of various incubation time were evaluated.

EXPERIMENTAL

Materials and apparatus

All the chemicals used in this study were of such quality that they did not require further purification. Tryptic Soy Broth, Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA) were purchased from Merck (Darmstadt, Germany). UV-Vis spectrophotometer (Shimadzu UV-1601) was used to record the absorbances of bacterial growth. *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and *K. pneumoniae* were obtained from Refik Saydam Hıfzıssıhha Centre (Ankara/Turkey). Sterile Petri dishes were supplied from Labkon Ltd (Turkey).

METHODS

SWO method

SWO method, which was described in detail in the previous study, is summarized below.[2] Wastewater taken from the agrochemicals production plant was purified from the mud and particles it contains and then diluted 5 times and used as a stock in this study. A home-made system consisting of a stainless-steel cylindrical reactor and a digital thermometer as subcritical water degradation equipment. In each experiment (hereafter referred as run), the amount of hydrogen peroxide determined according to the experimental program created by the Response Surface methodology, a chemometric method, was added to 150 mL of stock. The mixture was treated at the specified temperature under SWO conditions. At the end of the treatment time, treated samples were collected and kept at 277 K for further analysis

such as TOC removal, color removal and microbial activity analyses.

Bacteria and media

The wastewater indicator bacteria used for antibacterial activity and bacterial growth according to time screening were *E. coli*, *P. aeruginosa* and *K. pneumoniae.* The bacteria inoculum was prepared in 4 mL-Triptic Soy Broth medium and incubated at 37 ^{o}C, overnight. Then, the culture suspensions were adjusted to 0.5 McFarland Standard Turbidity (~10^8 for bacteria CFU per milliliter) and prepared daily and stored at +4 °C until use. Before, the experiments, all samples were filtered through a 0.45 µL filter.

Disc Diffusion Method

The disc diffusion method was employed to pre-examination for determination of the performance of treated and raw wastewater samples. Bacteria cultures (1.5 x 10^8) were spread onto MHA plates using drigalski spatula. Paper discs (6 mm in diameter) were impregnated on the agar to load 15 µL of samples and incubated at 37 °C for 24 hrs. The growth inhibition zones were measured.

Kinetic of bacterial growth

Kinetics of bacterial growth *Escherichia coli*, *K. pneumoniae* and *P. aeruginosa* were used for the kinetics growth test in treated and raw samples. To prepare the test culture, 0.3 mL of bacterial inoculum (suspended in 0.9% NaCl) was added into a 10-mL glass tube, which contained 5 mL of sterile MHB medium. Then, raw, run 13, run 14 and run 15 samples were added on the mixture in each case. Bacterial cultures without samples were prepared as a control. Optical densities were measured spectrophotometrically at 550 nm during 18 hours.

The evaluation growth kinetics of bacteria were modified based on Oscar's study (2000)[25]. The time-dependent growth curve was plotted by taking the averages of the recorded absorbance values. This semilogarithmic plot provides specific information about bacterial kinetics. In the plots, the lag time (lag-phase: t_{lag}) corresponds to the intersection of the horizontal line (no growth) and oblique line (exponential growth). The slope created according to the equation shows the specific growth rate of the bacteria. The value of "coefficient of x" in the equation (y = ax + b) can be considered as the specific growth rate (SGR).

RESULTS AND DISCUSSION

Although SWO is an effective and environmentally friendly method and provides striking results, microbial analysis of the treated samples obtained at the end of the process is of great importance for the reliability of the method.[5,26] Yabalak et al. mineralized oxacillin in the aqueous solution at the rate of 76.42% and subjected the treated and raw samples to antibacterial analyses. They demonstrated that the contaminated wastewater treated with SWO method shown less antimicrobial activity comparing to stock oxacillin solution.[5] In this study, the microbial activity potentials of run 13, run 14 and run 15 samples were evaluated and compared to those of the stock solution.

The compounds in raw wastewater, which were given in detail in the previous study, were significantly mineralized in run 13, run 14 and run 15. This finding was supported by high TOC and color removal and GC-MS results.[2]

Table 1

Experimental and predicted results of the TOC and color removals.[2]

Run	Temperature (K)	Concentration of H_2O_2 (M)	Treatment time (min)	TOC removal, %		Color removal, %	
				Exp.	Pre.	Exp.	[2]Pre.
13	373	1.2	50	45.89	44.99	97.85	98.21
14	433	1.2	100	59.45	58.32	97.92	97.28
15	403	0.8	75	49.22	49.77	92.38	93.39

Exp.: Experimental; Pre.: Predicted

Table 1 demonstrates the experimental and predicted results of the TOC and color removals of run 13, run 14 and run 15.[2] These runs, which provides removal rates from low to high levels, were selected to evaluate the antimicrobial activity analysis. In run 14, about 60% of TOC removal and 98% of color removal were obtained at 433 K in 100 minutes of treatment time using 1.2 M of H_2O_2. Beside run 14, two more levels (run 13 and run 15) were selected for further analysis. In these runs, TOC and color removals were obtained between 46%-50% and 92%-98%, respectively. In this manner, in addition to the effects of different TOC and color removals, the effects of different temperatures, oxidant concentrations and treatment time on the antimicrobial activities can be examined in more detail.

Table 1 demonstrates the results of the 3 runs from the total 20 runs of the above-mentioned study. Besides, the predicted results of the runs (13, 14 and 15) were in a good fit with the experimental results.

The SWO method of the degradation of the agrochemical wastewater was widely evaluated and optimised in the previous study using central composite design.[2] The statistical analysis was done using ANOVA to demonstrate the precision of the applied method.[2] Hence, it can be argued that the selected samples above-given (run13, run 14 and run 15) represent all other samples and the results obtained in the present study were binding for all samples.

Yabalak and Gizir reported that almost all compounds detected in the raw sample were degraded in the run 13 and run 14, except 2.4-di-*tert*-butylphenol, methyl palmitate and octadecanoic acid.[2] They attributed the inability of these three compounds to be degraded effectively to their straight-chain, highly alkylated and oxidation-resistant structure. According to GC-MS results, since no compound likely to exhibit microbial activity could be detected, no significant microbial activity could be detected in any run. Besides, raw wastewater sample showed higher microbial activity.

Evaluation of the pre-examination results and kinetics of bacterial growth

The inhibition effects of the treated and raw samples were done on bacteria before bacterial growth kinetic test using disc diffusion test. Any growth inhibition zone (mm) on bacteria during incubation with samples was not observed. Based on these findings, it can be said that the treated or raw samples have no antimicrobial effect. Growth kinetics of the investigated bacteria in the presence of raw, run 13, run 14 and run 15 samples were shown in Fig. 1. Measurable growth increase in the presence of treated and raw samples for *Escherichia coli, K. pneumoniae* and *P. aeruginosa* cultures was observed. The SGR and times of the t_{lag} were presented in Table 2.

Table 2

Growth kinetic parameters of *E. coli*, *K. pneumoniae* and *P. aeruginosa* in the cultures with the investigated raw, run 13, run 14 and run 15 samples according to the exponential growth model

	E. coli		***K. pneumonia***		***P. aeruginosa***	
	SGR	t_{lag}	**SGR**	t_{lag}	**SGR**	t_{lag}
Control	y = **0.0099**x + 0.0598	2	y = **0.003**x + 0.0492	2	y = **0.0032**x + 0.0702	2
Raw	y = **0.005**x + 0.0551	2	y = **0.0044**x + 0.0474	7	y = **0.002**x + 0.054	2
Run 13	y = **0.0026**x + 0.0616	2	y = **0.0032**x + 0.0371	8	y = **0.0029**x + 0.0512	2
Run 14	y = **0.0074**x + 0.0631	2	y = **7E-05**x + 0.0489	>18	y = **0.007**x + 0.0399	2
Run 15	y = **0.0036**x + 0.0644	2	y = **0.0036**x + 0.0705	2	y = **0.0049**x + 0.0392	4

SGR: specific growth rate. **$t_{lag\ (h)}$:** the end of lag phase (beginning of exponential growth phase).

The SGR of control bacteria cultures were characterized at about 0.0099x, 0.003x and 0.0032x levels for *Escherichia coli*, *K. pneumoniae* and *P. aeruginosa,* respectively. The SGR values of cultures in raw, run 13, run 14 and run 15 samples were measured between 0.0026x and 0.005x for *Escherichia coli* (Fig. 1a) while they were 0.002x and 0.007x for *P. aeruginosa* (Fig. 1c). In *K. pneumonia* (Fig. 1b), SGR parameters were 0.0044x, 0.0032, 7E-05x, 0.0036x in raw, run 13, run 14 and run 15 samples. It was noted here that the growth of *K. pneumoniae* treated with run 14 is very low (7E-05x) because of its inhibitory effect. Addition of the treated samples to the *K. pneumoniae* culture media resulted in the reduction of the SGR. The SGRs

can be presented as: r14>raw>r15>r13 for *E. coli*; r14>r15>s>r13>raw for *P. aeruginosa*, raw>r15> r13>r14 for *K. pneumoniae* at the end of the 24 h incubation ($p \leq 0.05$). In *E. coli*, t_{lag} phases in raw, run 13, run 14 and run 15 added conditions were 2 hours. Similarly, t_{lag} was 2 hours in raw, run 13, run 14 for *P. aeruginosa*, but 4 hours in run 15. Differently, t_{lag} for *K. pneumoniae* in raw, run 13, run 14 and run 15 were 7, 8, >18, 2, respectively (Fig. 1).

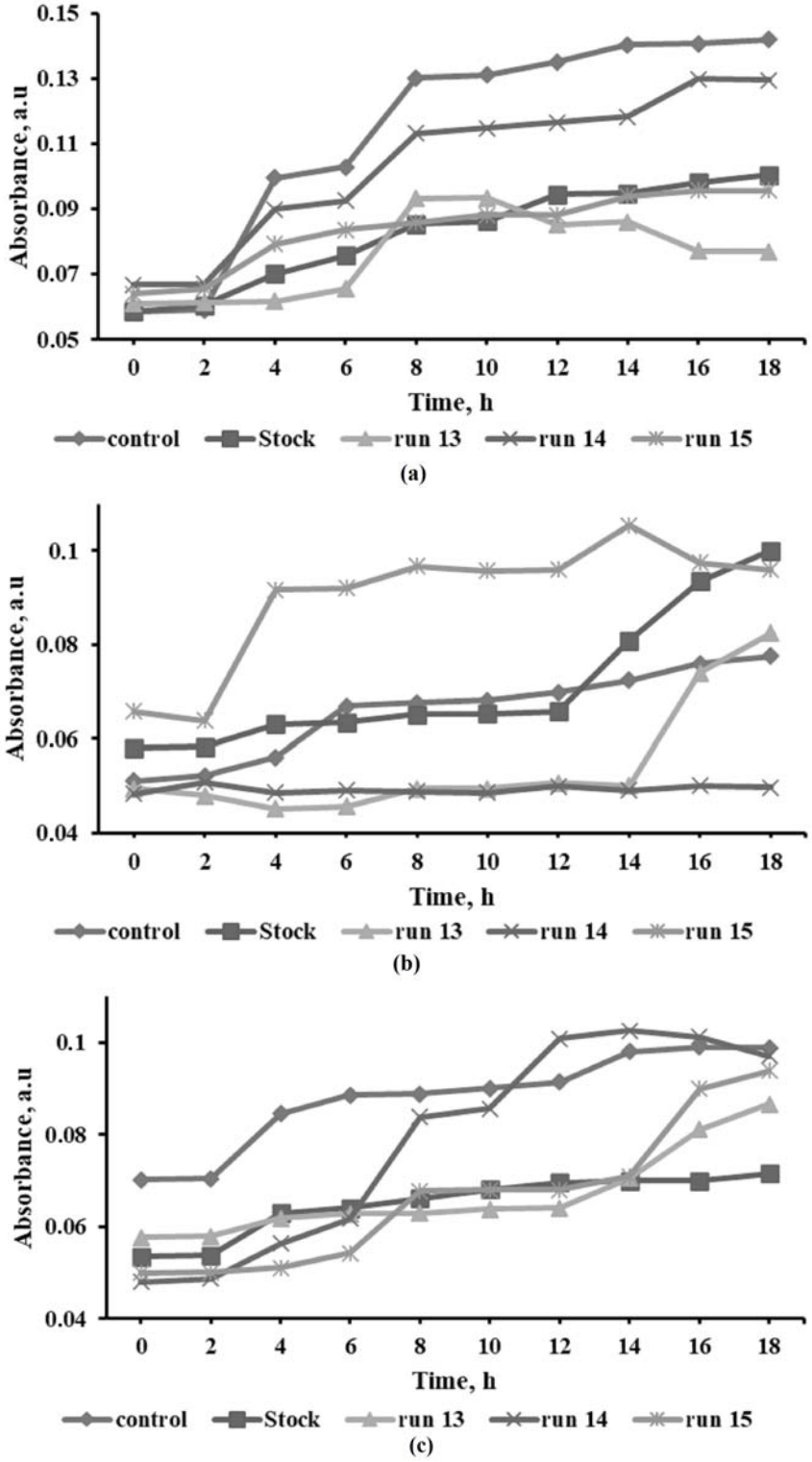

Fig. 1 – Growth of (a) *E. coli*, (b) *K. pneumoniae* and (c) *P. aeruginosa* in cultures with the investigated raw, run 13, run 14 and run 15 according to the exponential growth model.

Organic carbon is an energy substrate for aquatic organisms as well as stimulating the growth of pathogenic microorganisms. The main reason for this is the amount of dissolved oxygen in water increases due to the increase of organic carbon sources.[27] To a certain extent, this increase has been shown to stimulate proliferation for wastewater indicator microorganisms such as *E. coli*,[28] *K. pneumoniae*[29] and *P. aureginosa*.[30] In this study, the reproductive status of these pathogens in both raw and treated samples of organic carbon was evaluated. The findings remarkably showed that the development of *K. pneumoniae* slows down when the color and organic content of wastewater decreases. This was not observed in the other two bacteria, *E. coli* and *P. aureginosa*, where a previous study focused that coliforms live even at low carbon levels.[31] Finally, it was obtained that the growth rate of bacteria in the treated samples' medium did not increase more than the control and the growth of *K. pneumoniae* an important pathogen, slowed down. Besides, it can be said that the amount of organic content that can encourage bacterial growth may decrease with the treatment of wastewater.

CONCLUSIONS

Treated wastewater is likely to threaten human health, as it promotes the formation or reproduction of various pathogens. The degradation by-products that may occur after the treatment of wastewater have the potential to cause various health problems. Therefore, monitoring microbial activity is of great importance. Microbial activity of the industrial agrochemical production plant wastewater containing a large number of hazardous compounds and ions with high amounts, which has been previously treated by removing the total organic carbon (59.45%) and color (97.92%) using the subcritical water oxidation (SWO) method, was analysed in detail. Generally, it can be stated that the growth parameters of *E. coli* and *P. aureginosa* are similar for the control and raw, run 13, run 14 and run 15, but it was seen that specific growth rate of *K. pneumoniae* was significantly different in the media with raw, run 13, and run 14 than the media with run 15 and control. Finally, it was remarkable that run 14 sample strongly slows down the growth rate on *K. pneumoniae*. Especially, the SWO method can be applied to the real wastewaters of the hospitals where *K. pneumoniae* is more frequently detected.

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