KLEBSIELLA PNEUMONIAE DISINFECTION WITH ULTRASOUND AND HYDROGEN PEROXIDES

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Abstract. Water-borne epidemics caused by pathogenic microorganisms endanger public health especially in countries where access to clean water sources is difficult. Also, the conventional disinfection methods have various disadvantages on environmental health and they can be ineffective against some pathogenic microorganisms. For these reasons, the disinfection process applied in water has gained great importance and new alternative treatment technologies have to be developed to replace traditional methods used to protect public health. In this study, the effects of ultrasound (US) and hydrogen peroxide (H_2O_2), which have been used for water disinfection in recent years, have been examined whether or not they can remove *Klebsiella pneumoniae* from water. This study has concluded that the combination of US and H_2O_2 can effectively remove *Klebsiella pneumoniae* bacteria from water. When the application of ultrasound was performed with a H_2O_2 concentration of 5 mg l^{-1}, the disinfection efficiency of *Klebsiella pneumoniae* was 4.22 log after 60 min.
Keywords: *water disinfection, ultrasound, hydrogen peroxide, coliform*

Introduction

Water is significantly contaminated by human or other environmental factors. Water, which is required for the survival of living organisms, are polluted by anthropogenic sources, such as, chemical, physical and microbial contaminants. Especially, water storage tanks can be contaminated significant pollutants, such as leaks, mixing of sewage sludge from soil cracks, agricultural runoffs, unsuitable industrial applications, mining applications, underground injection of waste chemicals, and corrosive waters (WHO, 2004, 2011). The legal regulations to ensure clean and reliable drinking and potable water must be very strict in application and control, and so clear and easy to update in terms of compliance with scientific developments (WHO, 2004, 2011).

Pathogens that cause waterborne diseases include; bacteria, viruses, protozoa and algae (Ashbolt, 2004; Moe, 2007). Water-borne epidemics diseases have significantly reduced by chlorinating drinking water since 1900 (Akin et al., 1982) but can be ineffective against to some pathogenic microorganisms. *Klebsiella pneumoniae* is stationary, non-spore, short, rounded, 1-2 μm in length and 0.5-0.8 μm in with basil. *Klebsiella pneumoniae* is best grown at 12-43°C and at pH 7 using bloody agar, endo agar and EMB agar media in their analysis, which can show capsular, aerobic and facultative anaerobic character in Gram negative polysaccharide structure. Because it is a bacterium found in the upper respiratory tract and stool flora in humans, pathogenicity is released as an opportunistic pathogen in the face of inappropriate conditions (Dutka, 1973). The sources of pathogenic microorganisms in drinking water are surface waters and groundwater contaminated with fecal matter (Shiddamallayya and Pratima, 2008; Gray, 2014). Drinking water supplies are contaminated with fecal *Klebsiella pneumoniae* via domestic wastewater, meat treatment processes wastewater and the surface flow from farms (Percival, 2014). In addition, *Klebsiella pneumoniae* are responsible for hospital

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infections (Percival, 2014). *Klebsiella pneumoniae* is listed as total coliform in the international guidelines (Arbuckle et al., 1976). As for standard of WHO, the number of total coliform in 100 ml of untreated ground water is limited to less than 10 cells (WHO, 2004), and it is indicated in the guideline that there should be no fecal coliform in water samples (LeChevallier and Au, 2013).

Because of the various disadvantages of conventional disinfection methods on the environment and human health, the use of advanced treatment methods for disinfection have gained importance with effective inactivation of pathogenic microorganism to prevent disinfection by-product formation in conventional disinfection methods especially (Sirivedhin and Gray, 2005). At the beginning of advanced treatment methods are ultrasound, Fenton oxidation, UV, ozone, hydrogen peroxide applications. UV oxidation technologies; (titanium dioxide) in a homogeneous environment by the addition of a suitable oxidizing agent (hydrogen peroxide, ozone) (Stasinakis, 2008; Koparal, 2018). Homogeneous processes (UV / H_2O_2, UV / O_3) and heterogeneous processes (photolysis of semiconductor particles) as an advanced treatments methods also used for water disinfection but these alternative methods has some limits (Venkatadri and Peters, 1993). For examples, UV disinfection equipped with lamps producing 254 nm wave length UV rays is used to inactivate the microorganisms in the water, but it is difficult to obtain effective inactivation ratio in water with high turbidity (Qualls et al., 1983; Christensen and Linden, 2003; WHO, 2004). Also ozone application needs addition of chlorine after effective disinfection in order to supply residue protection (Langlais et al., 1991).

Hydrogen peroxide (H_2O_2) is a powerful chemical disinfection agent (Falagas et al., 2011). Disinfection efficacy of H_2O_2 depends on its concentration, contact time and microorganism (Alasri et al., 1992). H_2O_2 has wide-ranging activity against viruses, bacteria, yeast, bacteria spores. However, the effect of hydrogen peroxide is more effective against gram-positive, rather than gram-negative bacteria. Hydrogen peroxide is a powerful oxidizing agent because it produces free hydroxyl radicals. High-level disinfection can be obtained with hydrogen peroxide in the critical patient care units (Arrage et al., 1993).

Ultrasound or, in other words sonication, is one of the alternative advanced treatment technologies for water disinfection (Stasinakis, 2008). The inactivation of microorganisms depends on the time and power of the ultrasonic application. The ultrasound effect on bacteria is based on the destruction of the cytoplasmic membrane (Mason et al., 2003). When the ultrasonic wave passes through a liquid, very small bubbles are formed and this is called cavitation. The collapse of these cavitation bubbles creates high temperatures and pressures locally. These sudden changes in temperature and pressure cause the cell wall structure to deteriorate. When the US is combined with conventional chemical treatment, it increases the potency of the oxidizing chemical in the microbial cell membrane (Jatzwauk et al., 2001). Factors affecting ultrasound removal efficiency are temperature, wave frequency, wave amplitude, applied power, medium viscosity, contaminant type, surface tension of solution and solution vapor pressure (Mason and Peters, 2002).

In this study, the synergistic effects of ultrasound (US) and hydrogen peroxide (H_2O_2) have been examined on the removal of *Klebsiella pneumoniae* from water in order to eliminate the limited applications of these two methods. It has been shown that the combination of US and H_2O_2 can effectively remove *Klebsiella pneumoniae* bacteria from water.

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Materials and Methods

This research was conducted in Department of Environmental Engineering, Faculty of Engineering, Eskisehir Technical University, and Eskisehir, Turkey. In this study, the disinfection efficiencies of ultrasound (US), hydrogen peroxide (H_2O_2) and hybrid application of these methods.

Microbiological studies

Klebsiella pneumoniae working solution of 100 ml and a concentration of 1×10^5 CFU ml^{-1} was disinfected at three ultrasonic frequencies and three H_2O_2 concentration. The disinfection studies were performed in a sterile cabinet (Heraeus KSP-18 Class II) at room temperature. The bacterial concentration was determined with a simple plate count method during all disinfection studies using serial dilution of the samples. All the samples were inoculated on plate count agar (PCA, Merck-Germany) solid media, and the plates were incubated at 37°C for 18-24 hours (Karel, 2016). The bacterial inactivation ratio was calculated as the difference between the initial bacterial concentration and final bacterial concentration for each disinfection study. The disinfection efficiencies of systems were determined with the average bacterial concentration of samples taking into account *Eq. 1* accounting to logarithmic term.

$$E\ (log)\ =\ log\ (Co - Ct) \qquad \text{(Eq.1)}$$

where:
E (log) = Disinfection efficiency in logarithmic term,
C_0 = Initial average bacterial concentration (CFU ml^{-1}),
C_t = Average bacterial concentration at t time (CFU ml^{-1}).

The results of microbiological studies were expressed as average of three independent experiments with error bar using standard deviation (SD). Each experiment was performed with three parallels. Statistical significance of the difference of the counted bacteria in in plate count agar among each trials was determined by analysis of variance (ANOVA) using two-way test. In order to show reliability of measurement, p value obtained from two-way ANOVA test was given for each disinfection study.

Ultrasonic disinfection studies

Disinfection studies were carried out in a W-113 Ultrasonic multi-cleaner (Honda Electronics Co., Ltd., Aichi, Japan) given in *Fig. 1* using batch flow condition. This batch type of ultrasonic reactor was operated at ultrasonic frequencies of 28, 45 and 100 kHz and at a power of 100 W. Ultrasonic disinfection studies were performed for a contact time of 60 minutes.

Disinfection with hydrogen peroxide

Hydrogen peroxide is a strong oxidizing agent and has a bacterial inactivation effect. The bacterial inactivation effect of hydrogen peroxide on *Klebsiella pneumoniae* was examined for concentrations of 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} in the working solution. Hydrogen peroxide stock solution of 500 mg l^{-1}, 1000 mg l^{-1} and 2000 mg l^{-1} were freshly prepared before the hydrogen peroxide disinfection studies. These working concentrations of hydrogen peroxide (5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1}) were set in the

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working solution using 1 ml of suitable stock solution of each concentration. The disinfection studies were performed with three different concentrations of hydrogen peroxide, and the effect of bacterial inactivation was determined in the same working solution at similar flow conditions and contact time.

Figure 1. *Batch type of ultrasonic reactor was operated at ultrasonic frequencies of 28 kHz, 45 kHz and 100 kHz*

Disinfection with hydrogen peroxide and ultrasound

The hybrid disinfection studies of ultrasound and hydrogen peroxide were conducted in an ultrasonic reactor with H_2O_2 concentrations of 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} and applying a 28 kHz ultrasonic frequency. To determine the synergetic effect of H_2O_2, the US disinfection studies were repeated with similar conditions as the previous disinfection process.

Results and discussion

The effect of ultrasonic frequency on *Klebsiella pneumoniae* disinfection process at an average of 1×10^5 CFU ml^{-1} is shown in *Figure 2*.

When a 28 kHz ultrasonic frequency was used to inactivate *Klebsiella pneumoniae*, a 3.52-log disinfection efficiency was attained with a 60-min disinfection period. When a 45 kHz ultrasonic frequency was used to inactivate *Klebsiella pneumoniae*, a 1.52-log disinfection efficiency was attained with a 60 min disinfection period. When a 100 kHz ultrasonic frequency was used to inactivate *Klebsiella pneumoniae,* a 1.1-log disinfection efficiency was attained with a 60 min disinfection period. The results of ultrasonic disinfection studies showed that the maximum bacterial inactivation rate at an ultrasonic frequency of 28 kHz.

In the present study, the highest inactivation efficiency of *Klebsiella pneumoniae* was determined at an ultrasonic frequency application of 28 kHz after a 60-min disinfection

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period as the bacterial inactivation ratio is dependent on the mechanical disruption effect on the cell membrane from ultrasonic frequency. Gao et al. (2014b) studied *Enterobacter aerogenes, Bacillus subtilis, Staphylococcus epidermidis, S. epidermidis* and *Staphylococcus pseudintermedius* microorganisms at ultrasonic frequency 20 kHz and 100 kHz applying 13 W power. It was observed that the highest bacterial inactivation rate was determined to be 4.5-log after 20 min continuous sonication at 20 kHz ultrasonic frequency differing microorganisms(Gao et al., 2014a).

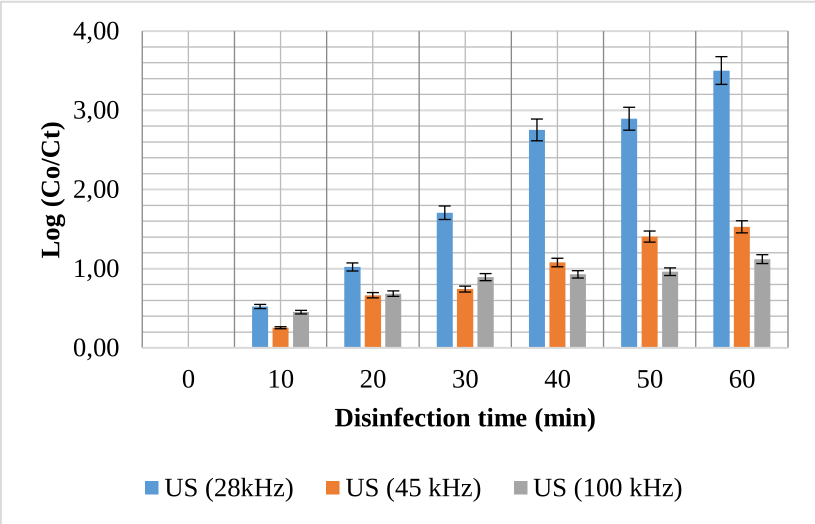

Figure 2. *Effect of ultrasonic frequency on the Klebsiella pneumoniae disinfection process at a concentration of 1 × 10^5 CFU ml^{-1} (p value was calculated as 0,334 using two-way ANOVA test. This value showed that there is no significant difference of each measurements among each three disinfection experiment because p>0.01)*

This ultrasonic frequency effect was disgusted in previous research(Gemici et al., 2014; Karel, 2016) by high-power ultrasound frequencies, which are described as using low ultrasonic frequencies between 16 kHz and 100 kHz because high-power ultrasound is more effective than high-frequency (500 kHz-1 MHz) ultrasound when performing bacterial inactivation(Joyce et al., 2003). The chemical effects of high-frequency ultrasound used in this study leading to thermal decomposition of water molecules into OH^- and H^+ radicals was probably insufficient for the inactivation of *Klebsiella pneumoniae,* although the physical effect of low-frequency ultrasound enhanced the efficiencies of bacterial inactivation(Mason et al., 2003).

The result of hydrogen peroxide disinfection studies is shown in *Fig. 3*. When a 5 mg l^{-1} $H_2O_2^+$ concentration was used to inactivate *Klebsiella pneumoniae*, a 0.04-log disinfection efficiency was attained with a 60-min disinfection period. When a 10 mg l^{-1} H_2O_2 concentration was used to inactivate *Klebsiella pneumoniae*, a 0.04-log disinfection efficiency was attained with a 60 min disinfection period. When a 20 mg l^{-1} $H_2O_2^+$ concentration was used to inactivate *Klebsiella pneumoniae*, a 0.08-log disinfection efficiency was attained with a 50 min disinfection period. These results showed that increasing the hydrogen peroxide concentration raises the bacterial inactivation ratio as expected (Loraine et al., 2012), however, the they indicated that obtained inactivation ratio of *Klebsiella pneumoniae* was not enough for effective disinfection.

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The disinfection study performed in an ultrasonic reactor with addition of 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1} of H_2O_2 during a 60 min disinfection period under 28 kHz ultrasonic frequency is shown in *Fig. 4* and Anova: two-way test results of final disinfection study were summarized in *Table 1*.

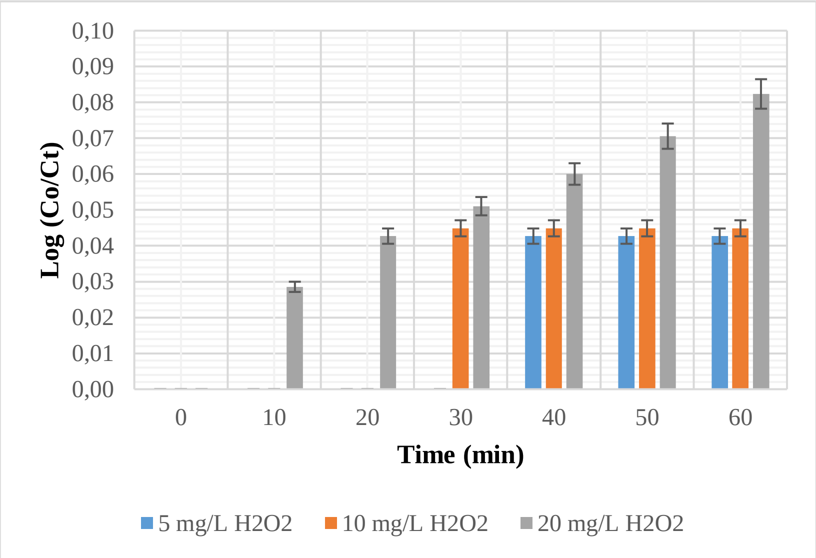

Figure 3. *Effect of hydrogen peroxide concentration on the Klebsiella pneumoniae disinfection process (5, 10 and 20 mg l^{-1} H_2O_2; 1×10^5 CFU ml^{-1} of Klebsiella pneumoniae and 60 min disinfection period under batch conditions) (p value was calculated as 0,000000009 using two-way ANOVA test. This value showed that there is a significant difference between and within of hydrogen peroxide disinfection experiment because $p<0.01$)*

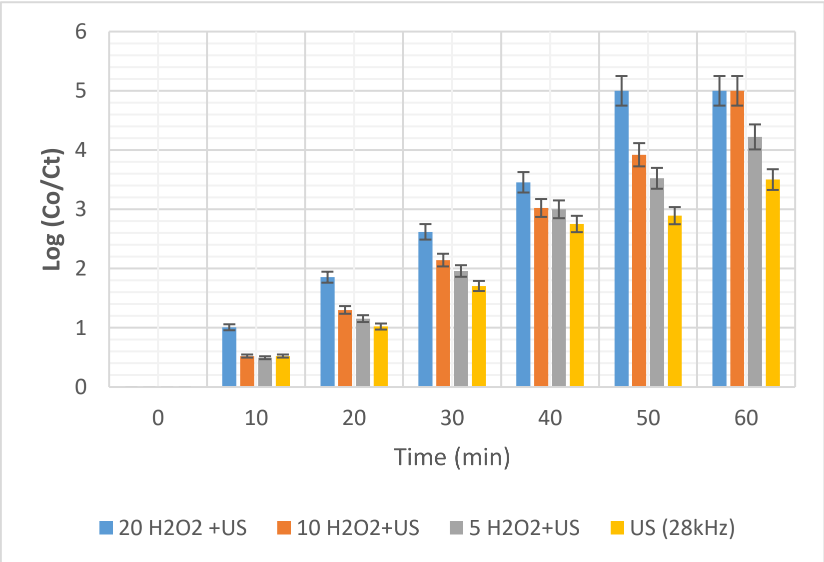

Figure 4. *Effect of hydrogen peroxide concentration on the ultrasonic disinfection process (an ultrasonic frequency of 28 kHz and 100 W power at 5, 10 and 20 mg l^{-1} H_2O_2; 1×10^5 CFU ml^{-1} Klebsiella pneumoniae and 60 min disinfection period under batch conditions) (p value was calculated as 0,071 using two-way ANOVA test. This value showed that there is no significant difference between and within of hydrogen peroxide disinfection experiment because $p>0.01$)*

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Table 1. *Anova table and descriptive statistical values of the effect of hydrogen peroxide concentration on the ultrasonic disinfection process*

ANOVA: TWO-WAY				
Summary	*Number*	*Total*	*Average*	*Standard deviation*
***Trial* 1**	5	100407	20081.4	2E+09
***Trial* 2**	5	100538.3	20107.65	1.99E+09
***Trial* 3**	5	120645.9	24129.18	2.87E+09
Initial bacteria concentration	3	320000	106666.7	1.33E+08
20 mg l^{-1} H_2O_2 + US (28kHz)	3	119.7	39.9	32.83
10 mg l^{-1} H_2O_2+ US (28kHz)	3	335.35	111.7833	330.6158
5 mg l^{-1} H_2O_2+ US (28kHz)	3	375	125	625
US (28kHz)	3	761.1	253.7	5117.07

ANOVA- *descriptive statistical values*						
Standard deviation Resources	*SS*	*df*	*MS*	*F*	*P-value*	*F -criteria*
In groups	54263193	2	27131596	1.02183	0.402524	4.45897
Between groups	2.72E+10	4	6.81E+09	256.4678	1.78E-08	3.837853
Error	2.12E+08	8	26551961			
Total	2.75E+10	14				

When the application of ultrasound was performed with a H_2O_2 concentration of 5 mg l^{-1}, 10 mg l^{-1} and 20 mg l^{-1}, the disinfection efficiency of *Klebsiella pneumoniae* was 4.22 log after 60 min, 5 log after 60 min and 5 log after 50 min, respectively. This indicates that *Klebsiella pneumoniae* disinfection with hydrogen peroxide and ultrasound decreased the disinfection period and increased inactivation ratio according to a single hydrogen peroxide application. The physical effects of low-frequency ultrasound improved the bacterial inactivation ratio by enhancing the disinfection effect of hydrogen peroxide through synergistic effects of ultrasound. The results of microbiological studies were expressed as average of three independent experiments with error bar using standard deviation (SD) using three parallels studies of all disinfection process. The results of analysis of variance (ANOVA) using two-way test, p value was calculated 0,402524 within groups and p value was calculated 0,0000000178 between groups These value showed that there is no significant difference within groups of each three disinfection experiment because p value was greater than 0.01. To concluded, two-way ANOVA test indicated that US+ H_2O_2 hybrid applications gave reliable and repeatable results.

Image of *Klebsiella pneumoniae* on petri dish from disinfection with hydrogen peroxide and ultrasound studies was given in *Fig. 5* as initial bacteria concentration in *Fig. 5A*, 5 mg l^{-1} H_2O_2+ US 28 kHz in *Fig. 5B*, 10 mg l^{-1} H_2O_2+ US 28 kHz in *Fig. 5C* and, 20 mg l^{-1} H_2O_2+ US 28kHz in *Fig. 5D*. These results indicate that ultrasound and hydrogen peroxide reduced the hydrogen peroxide concentration to obtain an eligible bacterial inactivation efficiency in water because ultrasound accelerates the contact of OH^- and H^+ radicals, which are the decomposition products of H_2O_2, with bacteria.

Ultrasound can also facilitate breakage of microorganism agglomeration, thereby increasing the efficiency of other chemical disinfectants (Karel, 2018). This effect was clearly shown in literature. Gao et al. (2014a) was studied disinfection of *E. aerogenes*, *Bacillus subtilis*, *S. epidermidis* and *A. Pullulans* with hydrogen peroxide (30%) and t-butanol in 85 kHz ultrasonic reactor. As a result of their study, it was observed that the addition of H_2O_2 provided microorganism inactivation much shorter than ultrasound disinfection (Gao et al., 2014b). Lakeh et al. (2013), was disinfected *Anguillicola crassus*,

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Paramecium sp. and *Artemia sp*. with low-frequency US and UV-C methods. They found that the mean size of suspended solids in the water culture with low frequency US decreased and therefore the UV-C inactivation rate increased by 0.6 log. Comparison of UV-C radiation with low-frequency US did not cause a decrease in the number of colonies in fresh water, but the antiseptic effect of UV-C in turbid water increased by 0.6 log. When colony count values were compared only with UV-C and low-frequency US with UV-C, pretreatment with low frequency US showed significant effect. The US sonication of 0.2-18.8 kJ/L low frequency dose range varies from 7% to 95% depending on the inactivation rate of paramecium (Lakeh et al., 2013). Ayyıldız et al. (2011) have demonstrated the effects on *E. coli* and total coliform inactivation when used in combination with chlorine dioxide (ClO_2, 2 mg /L) and US (150-300 W/L) in wastewater disinfection. Raw waste water and synthetic water are used. US (vibra cell 505) 500 W power and 20 kHz frequency were used. The log removal was 1.4-1.9 in single treatments and the combined use of consecutive US (150 or 300 W/L) and ClO_2 (2 mg/L) resulted in 3.2 - A 3.5-month fix was provided. Using the US and ClO_2 disinfection methods together, high concentrations of particles in the raw wastewater and their breakdown under shock noise waves have been achieved (Ayyildiz et al., 2011).

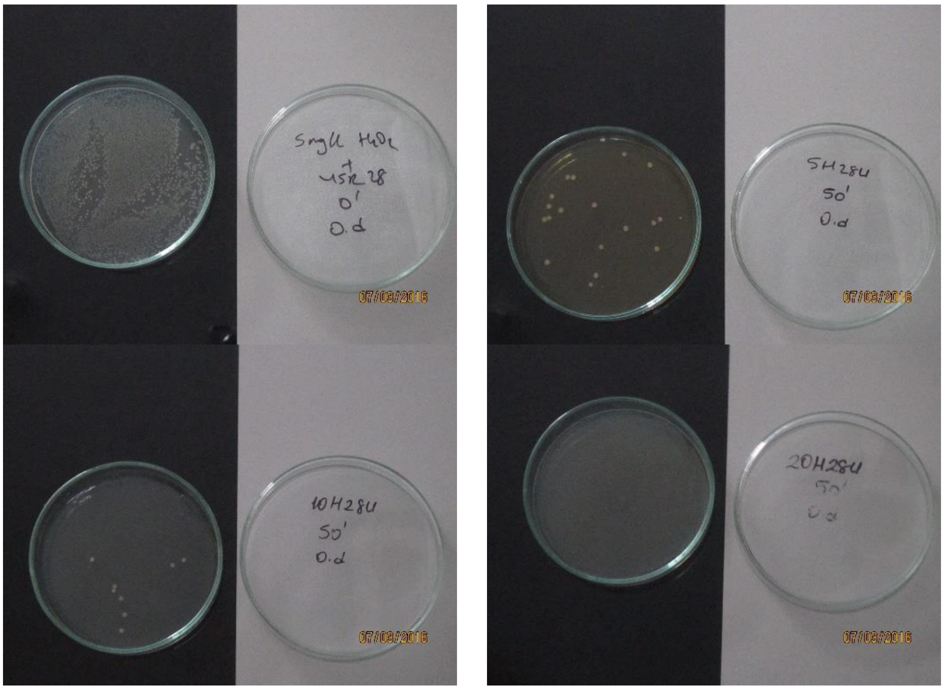

Figure 5. *Image of Klebsiella pneumoniae on petri dish from disinfection with hydrogen peroxide and ultrasound studies (A: initial bacteria concentration, B: 5 mg l^{-1} H_2O_2+ US 28 kHz, C: 10 mg l^{-1} H_2O_2+ US 28 kHz, D: 20 mg l^{-1} H_2O_2+ US 28kHz)*

Hydrogen peroxide, which has a significant effect on the disinfection performance of the ultrasound, can be generated simultaneously during the treatment of water and wastewater depending on the conditions under which the treatment takes place and the ultrasonic system parameters (frequency, power, amplitude, power density, etc.). This is most likely to be the production of OH-H radicals at ultrasonic frequencies in the range of 100 kHz to 1 mHz and the sonochemical process called H_2O_2 oxidation(Suslick, 1988), but it is seen in the literature that high power and retention times should be applied for

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the production of these radicals (Rae et al., 2005). In this study, H_2O_2 could not be produced by using effective sono-chemically at the 28 kHz ultrasonic frequency, US-H_2O_2 disinfection studies were also showed the effect of H_2O_2 addition to the ultrasonic system.

This study was showed that ultrasound and its hybrid application with hydrogen peroxide. It has been seen that, when US combined with H_2O_2 with the accelerator effect of ultrasound on mass transfer as seen in other oxidation methods, bacterial inactivation ratio increased with less chemical usage. Also, the disadvantages of ultrasound eliminated with H_2O_2 hybrid studies because it requires high energy density for high inactivation ratio by the use of ultrasound alone. Due to the catalysis effect of the ultrasound, a significant reduction in the amount of H_2O_2 required for the disinfection resulted in H_2O_2 disinfection for high bacterial removal efficiency. As a result of the study, it has been determined that the *Klebsiella pneumoniae* in water and other ecosystem effects on human health can be successfully treated with hybrid US- H_2O_2 process.

Conclusion

The effects of different frequencies and added H_2O_2 on microorganism removal performance in ultrasonic system were investigated in this paper. It is clearly stated that this study will be an important development in drinking and potable water disinfection to reduce limitation of these alternative methods. It was concluded that water could be microbiologically treated by ultrasound providing less energy consumption and less chemical usage. Microorganism inactivation can be recommended in higher yields by employing the ultrasonic system as a hybrid. The studies have been carried out in a small scale in the laboratory environment and this study can be given as a result of this study in pilot scale and larger systems. It can also be tested in the ultrasound system with separate studies for other microorganisms that can be found in the targeted water environment.

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