Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2018.22211

119 (2018) 36–43
July

Comparison of the efficiency of natural and synthetic zeolites modified with cationic surfactants as a disinfectant against *Escherichia coli*, *Enterobacter*, and *Enterococcus*

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Received 19 September 2017; Accepted 9 March 2018

ABSTRACT

The aim of this study was to compare the efficiency of modified zeolite with two cationic surfactants as a disinfectant against *Escherichia coli*, *Enterobacter*, and *Enterococcus*. Natural and synthetic zeolites were modified by cetyltrimethylammonium bromide (CTAB) and cetylpyridinium chloride by the impregnation method. For modification of zeolites, surfactants were used equal to 0.5, 1.0, and 2.0 ECEC of each zeolite. The examined bacteria were isolated from polluted water. Bacteriological examinations were conducted by microbroth dilution and agar disc diffusion (Kirby–Bauer) methods. The results showed that Gram-positive bacteria are more sensitive than Gram-negative bacteria. Additionally, the antibacterial activity of modified natural zeolite was better than synthetic zeolite (p = 0.02). Also, significant differences between the inhibition zone of natural zeolite and synthetic zeolite values with parent zeolite were observed (p = 0.003 and p = 0.018, respectively). *Enterococcus* and *Enterobacter* show the highest efficiency for CTAB-natural zeolite with an inhibition zone diameter of 15 ± 3.6 and 9.33 ± 8.08 mm, respectively. The microbroth dilution test showed that Gram-negative bacteria are resistant against modified natural zeolite and modified synthetic zeolite with cell density of 1.5×10^8 CFU/mL and $\leq 3 \times 10^8$ CFU/mL, respectively, while Gram-positive bacteria show nearly 0 cell density. It is concluded that modified zeolites could have effect on water indicator bacteria.

Keywords: Antibacterial activity; Cationic surfactant; Indicator bacteria; Modified zeolite

1. Introduction

Nowadays, several studies have been done on the pollution in water, wastewater, and air that threatens human health [1–3]. Waterborne diseases are responsible for most of the mortality in developing countries worldwide. According to international reports, at least one-sixth of the world population does not have access to safe water, and that leads to the mortality of 2.2 million people, especially children under the age of 5 years, every year [4,5]. A specific group of microorganisms in water is responsible for creating a health risk; therefore, water disinfection and microbial control are necessary [6]. Indicator microorganisms have been recognized as pathogens in water since long ago [7,8]. Water indicator microorganisms can be easily detected by a simple method [4].

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Water quality management and health risk assessments have been evaluated by indicator bacteria including total coliforms, fecal coliforms, *Escherichia coli*, fecal streptococci, and *Enterococcus* [9,10]. For removing all groups of the pathogens such as *Cryptosporidium* and *Giardia*, an extremely high disinfectant dosage will be required. In addition, the bacteria resistance to conventional disinfectants has led researchers to explore a wide range of study to find new and harmless agents [11]. Zeolites are natural and harmless substances that consist of a three-dimensional arrangement of SiO_4 and AlO_4 with a shared oxygen atom between them. Also, internal surface areas have a permanent negative charge [12]. One of the most abundantly used zeolitic minerals in the world is the natural zeolite clinoptilolite [13]. There is a difference between natural and synthetic zeolite. Consequently, surfactants on the various zeolites show various behaviors to anions [14]. Among different quaternary ammonium compounds, cetyltrimethylammonium bromide (CTAB) and cetylpyridinium chloride (CPC) are the most commonly used cationic surfactants which have a long chain with a positive charge [15]. Crystal structures of zeolites have negative charges that make them good cation exchangers. The definite pore size of zeolite makes it suitable for modifying by large cationic surfactants [16] such as CTAB and CPC [17]. The concentration of surfactants in the modifying zeolites is important, and if it was above the critical micelle concentration, a double layer can be formed and completely adsorbed on the zeolite external surface [18]. This process leads to a charge reversal on the external zeolite surface from negative to positive [19]. On the other hand, zeolite modified by surfactants can be used as an effective anion exchanger. As it is known, absorption of surfactants on zeolite changes the surfactant arrangement. Meanwhile, morphology of zeolites plays an important role in the amount of loaded surfactant cations and particularly their arrangements. Several researchers have studied the modification of zeolite structure by cationic surfactants. Cationic surfactants have antimicrobial properties, which are considered as low-cost disinfectants, but have limitations due to high toxicity in using dosage [20,21]. Zeolites have pore size in the range of 5–7.5 Å, which is closer to the size of many molecules with pharmacological activity [22]. Taking this into account, zeolites have been used as an easier alternative and suitable drug delivers [23]. Modified zeolites are attractive candidates for various applications. In addition, modification of zeolites by surfactants has also showed different capacities for the removal of some organic compounds [24]. Surfactants on various zeolites show various behaviors to anions. In recent decades, several studies have been done on finding new disinfectants. For example, zeolites, as support for silver nanoparticle, have been studied to improve the antibacterial efficiency [25]. Also, antibacterial activity of natural zeolite containing various metal ions, such as silver, zinc, copper, mercury, cadmium, and chromium, was tested against bacteria [26–31]. There are several studies on the use of synthetic and natural zeolites supporting metal ions as bactericides for water disinfection [32]. However, there is no study, which compares the antibacterial activity of modified natural and synthetic zeolites on the aquatic environment. It was believed that the use of different zeolites as a supporter for surfactants has a major role on their antibacterial behavior. The aim of this study is to compare the efficiency of modified zeolite with two cationic surfactants, CTAB and CPC, as disinfectants against water indicator bacteria such as *E. coli*, *Enterobacter*, and *Enterococcus*.

2. Materials and methods

2.1. Reagents and chemicals

The synthetic zeolite (CBV 100) was supplied by Zeolyst International (China) with a SiO_2/Al_2O_3 mole ratio of 6:1; nominal cation in sodium form (Na_2O weight (%): 2.01). The unit cell size was 24.65 Å, and the surface area was 900 m^2/g. The external cation exchange capacity (ECEC) of zeolite was 0.53 Meq/g. Natural zeolite was purchased from Afrand Tuska C*ompany (Semnan, Iran)*. CTAB (≥98%), CPC monohydrate (>98%), and other analytical grade chemicals were purchased from Merck & Co. and used without further purification. Doubly distilled deionized water was used in total experiment process. The Gram-negative bacteria (*E. coli*) and Gram-positive bacteria (*Enterobacter* and *Enterococcus*) were isolated from polluted water. Barium chloride (Fluka, Buchs, Switzerland) and sulfuric acid (Fisher Scientific, Waltham, MA, USA) were used for McFarland preparation. All microbial cultures were obtained from Merck Company.

2.2. Apparatus

The X-ray diffraction pattern of modified zeolites was determined by Fourier transformation infrared (FTIR) spectroscopy using PerkinElmer spectrophotometer (PerkinElmer, USA). FTIR spectra of sample on KBr pellets were used for recording with a Nicolet beam FTIR spectrometer (PerkinElmer Spectrum 65; No. scan, 5; resolution, 8 cm^{-1}; scan speed, 0.2 cm/s; data interval, 2 cm^{-1}; detector, $LiTaO_3$) in the range of 400–4,000 cm^{-1} in room temperature [11]. Optical density (OD) measured with Jasco V-530 UV-Vis benchtop spectrophotometer (Jasco, Tokyo, Japan).

2.3. Modification of zeolites

CTAB-zeolite and CPC-zeolite were prepared by contacting each zeolite (synthetic and natural) with CTAB and CPC. For determining the effect of various concentrations of surfactants, 0.186, 0.364, and 0.729 g of CTAB and 0.02, 0.04, and 0.08 g (0.5, 1.00, and 2.00 CEC) of CPC were each added to natural and synthetic zeolites (2 g/L) [33,34]. The suspensions were stirred using a magnetic stirrer for 16 h at room temperature and filtered by a simple filtration technique. The solid residue was washed with distilled water twice, and solid samples were dried at 70°C overnight. The resultant products were readily used for antibacterial testing. Infrared (IR) spectroscopy was used for the structural analysis and the presence of surfactant molecules on zeolites.

2.4. Procedure for detection of Enterobacteriaceae family

For presumptive testing, the growth medium was lauryl tryptose broth, and all tubes were incubated at 37°C for 48 h and then examined for gas formation in the Durham tubes. Each positive tube was gently agitated, and a loop full of suspension was transferred to a tube of brilliant green bile

broth (BGLB) and EC *E. coli* broth. All the BGLB tubes were incubated at 37°C, and *E. coli* broth tubes were incubated at 45.5°C for 48 h. Any gas formation in the Durham tubes with turbidity in the media was regarded as a positive test. Then, the plates of eosin methylene blue agar were streaked with a loop full of suspension from the confirmed positive BGLB culture. Plates were incubated at 35°C for 18–24 h. Gram staining, microscopic examination, and biochemical tests, such as indole, methyl red, Voges-Proskauer, citrate, oxidative fermentative test, urea test, and other standard diagnostic tests, were used for selection and identification of bacteria. The resulting isolates were characterized morphologically and further identifications were carried out following the methods of Bergey's Manual of Determinative Bacteriology [35].

2.5. Procedure for detection of Enterococcus genus from water supplies

An inoculated series of tubes of azide dextrose broth was used with appropriate graduated quantities of samples with volumes of 10 mL or less at 35°C. Each tube was examined for turbidity at the end of 24–48 h. After 24 or 48 h of incubation, all azide dextrose broth tubes that showed turbidity would confirm the presence of *Enterococcus*. A portion of growth from each positive azide dextrose broth tube was streaked on Pfizer selective Enterococcus agar, and the plates were incubated at 37°C for 24 h. *Brownish-black colonies with brown halos confirm the presence of fecal strep*tococci. Then, *Enterococcus* bacteria were isolated by transferring brownish-black colonies "which are surrounded by black halo" to nutrient agar, and incubated for 24 h at 37°C. Then, brownish-black colonies with brown halos were transferred to two tubes of brain–heart infusion (BHI) broth: one with 6.5% NaCl and one without NaCl. When the tube was incubated at 37°C after 48 h (BHI broth with 6.5% NaCl) or 24 h (BHI broth without NaCl), the colony was confirmed as a member of the *Enterococcus* genus if growth was observed [36,37].

2.6. Antibacterial assay

2.6.1. Disc diffusion method

The antibiotic sensitivity pattern of isolated bacteria was determined on Mueller–Hinton agar plates by the Kirby–Bauer disc diffusion method according to National Committee for Clinical Laboratory Standards [38,39]. A sterile cotton swab was inserted into the bacterial suspension with turbidity equivalent of 0.5 McFarland standard (1.5×10^8 CFU/mL). The surface of the Muller–Hinton agar plate was inoculated with the swab. Modified and parent zeolites (0.01 g/L) were pressed into pellets (6 mm diameter). The plates were incubated at 37°C for 18–24 h. Antimicrobial activity was evaluated by measuring zone of inhibition (mm) after 24 h incubation time.

2.6.2. MIC method

The samples containing 1.5×10^8 CFU/mL bacterial suspension, 500 μL of medium, and 0.01 g of parent and modified zeolites were prepared and allowed to incubate at 37°C in 200 rpm. The blank, negative, and positive control solution was contained medium, medium with 1.5×10^8 CFU/mL bacteria, and medium with parent or modified zeolites, respectively. For determining minimal inhibitory concentration (MIC), OD of each sample was recorded by a UV-Vis spectrophotometer.

2.7. Statistical analysis

Experiments were conducted based on the mean ± standard deviation (SD). The antibacterial activity of modified zeolite against bacteria was analyzed by analysis of variance followed by the Tukey test. The differences between groups were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Characterization of zeolite-modified surfactant

FTIR spectra were used to confirm loading of surfactants (CTAB and CPC) onto the zeolites (natural and synthetic) surface. FTIR of modified natural zeolite by CTAB and parent zeolite is shown in Fig. 1. As it is clearly observable, the most important zeolitic materials come from stretching vibrations of SiO_4^{-4} and AlO_4^{-5} (600–1,200 cm^{-1}). The second (1,400–1,650 cm^{-1}) and third (3,000–4,000 cm^{-1}) group vibrations are because of deformation H–O–H molecules [40,41]. FTIR spectra of the modified zeolite show new peaks at 2,912, 2,849, and 1,478 cm^{-1}. The peaks are surfactants vibration that is related to the symmetric and the asymmetric stretching mode of C–H, C–C, and N–C bands, respectively [37,42]. The surfactants with long hydrocarbon tail chain "which has positive charge" play a major role in bonding to negative sites of

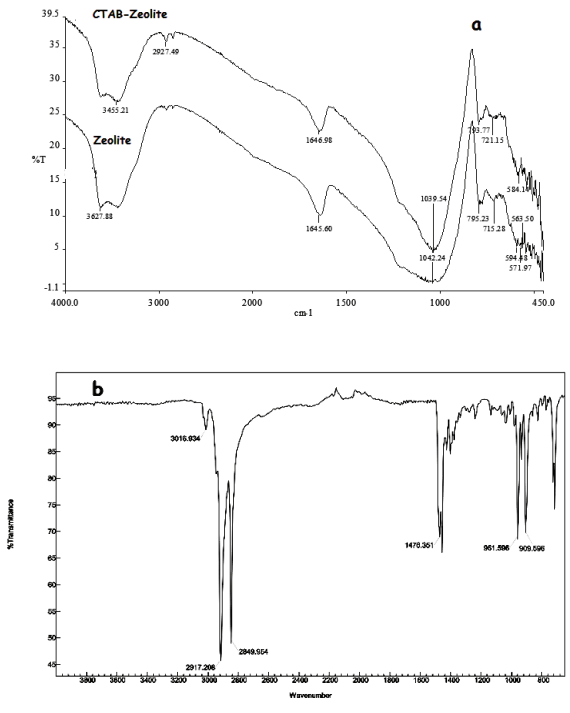

Fig. 1. FTIR patterns for natural zeolite and CTAB-natural zeolite (a) and cetyltrimethyl ammonium bromide (b).

zeolite. It indicates that the surfactant molecules have been successfully attached on parent zeolite [43,44]. This result is in agreement with other papers, which have examined zeolite modified by surfactant [45,46]. The IR spectra for synthetic zeolite, CPC, and CPC-zeolite are shown in Fig. 2. No relevant changes were observed in the structural vibration region of zeolite after absorption of CPC. This result confirms the structural stability of these composites. On the other hand, it indicates that the zeolite structure remains unaltered after the modification and that surfactant is present only at the zeolite surface [47].

3.2. Antibacterial assay

3.2.1. Disk diffusion method

The antibacterial activities of CTAB-modified zeolites and CPC-modified zeolites with different concentrations of surfactants were carried out by the disc diffusion (Kirby–Bauer) method, and the diameter of the inhibition zone was measured. The *E. coli* as Gram-negative bacteria and *Enterobacter* and *Enterococcus* as Gram-positive bacteria were examined in this study. Some selected images of the incubation-modified zeolites after 24 h are presented in Fig. 3. Inhibition zone diameters (mean ± SD) of modified zeolites (natural and synthetic) are shown in Table 1. The statistical analysis result shows that parent zeolites had nearly no inhibitory effect, whereas modified zeolite was effective against all the bacteria (p = 0.02) [48]. In addition, significant differences between the inhibition zone of natural zeolite and synthetic zeolite values with parent zeolite were observed (p = 0.003 and p = 0.018, respectively). The modified natural zeolite was the most effective against both Gram-positive and Gram-negative bacteria. Meanwhile, *Enterococcus* and *E. coli* were the most and the least sensitive bacteria, respectively. CTAB-zeolite (natural and synthetic) in 1.00 and 2.00 CEC shows significant antibacterial effect on the *Enterococcus* with an inhibition zone of 19 and 14 mm, respectively (Fig. 4). However, CPC-zeolite (synthetic) had more inhibition zone [10] in 2.00 CEC on the *Enterococcus* bacteria (Fig. 5). Several microbiological studies demonstrate that Gram-negative bacteria are more resistant than Gram-positive [49–52]. Disinfectant molecules must cross the outer layer of a cell to reach its target site. The nature and composition of this layer is special in various bacteria [53]. CPC-zeolite has the ability to change cellular membranes permeability, which can diffuse out intracellular ions and low molecular weight [54]. Intracellular depletion of bacteria results in consumption of available energy stores and cellular death [55]. The difference in the obtained result may be related to the intrinsic resistance of bacteria [56]. Bacteria have different cell responses in various conditions. Differences in the physicochemical characteristics of bacteria caused an electric charge at the cell surface. This electric charge can lead to the absorption and repulsion of ions. Fig. 6 illustrates the antibacterial mechanism of modified zeolites graphically. The synergy of antibacterial activity of modified zeolite can be affected by various mechanisms. Chook et al. [57] reported that the formation surface's positive charge is related to electron transfer rate and enhances the inhibition effect As well as modified zeolite can enhance the binding of bacterial DNA and block

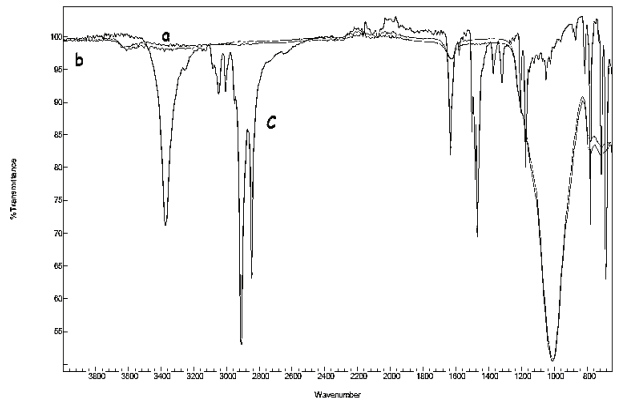

Fig. 2. FTIR patterns for CPC-synthetic zeolite (a), synthetic zeolite (b), and cetylpyridinium chloride (c).

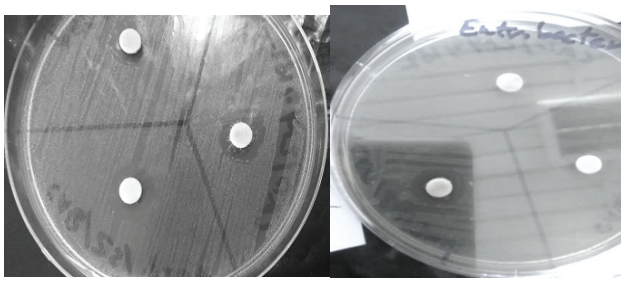

Fig. 3. Inhibition zone formed by the presence of CTAB-modified zeolite and CPC-modified zeolite (natural and synthetic) in the concentrations of 0.5, 1.00, and 2.00 CEC in the culture medium inoculated with *E. coli*, *Enterobacter*, and *Enterococcus* bacteria after 24 h of interaction.

Table 1
Antimicrobial activity of CTAB-zeolites (natural and synthetic) and CPC-zeolites (natural and synthetic) by the agar diffusion method

Inhibition zone (mm)	Cetyltrimethylammonium bromide			Cetylpyridinium chloride		
	Natural	Synthetic	Parent zeolite	Natural	Synthetic	Parent zeolite
E. coli	8.33 ± 7.23	5 ± 4.58	1.33 ± 1.15	4.33 ± 3.78	2.67 ± 4.61	0
Enterococcus	15 ± 3.6	13.67 ± 0.57	2.33 ± 2.08	1.67 ± 2.88	6 ± 5.29	0
Enterobacter	9.33 ± 8.08	8.33 ± 7.23	2 ± 1.7	3 ± 5.19	0	0

Note: Each value is the mean ± SD.

efflux pumps. Another suggested synergy "which is main advantage of this disinfectant" is preventing of bacteria from aggregation that greatly affects the antibacterial activities of modified zeolites [58]. The surfactants at low concentration form a monolayer on the zeolite surface. A second layer can be formed by increasing of loading surfactants on the zeolite surface. Bilayer is a surface with positively charged functional groups, thus creating absorption sites for anions [59]. Our results confirm this process and it was not an observed antibacterial activity for CPC-zeolite (natural and synthetic) in 0.5 CEC (as shown in Fig. 5). In this study, *E. coli* bacteria, which have an outer membrane, showed more resistance to the cell membrane damage.

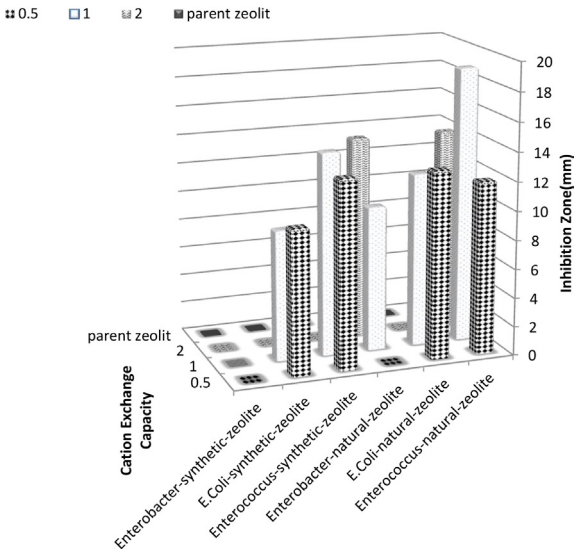

Fig. 4. Comparing the antibacterial activity of CTAB-zeolites (natural and synthetic) in the different CEC (0.5 CEC: 0.186 g, 1.00 CEC: 0.364 g, and 2.00 CEC: 0.729 g) against *E. coli*, *Enterobacter*, and *Enterococcus* bacteria.

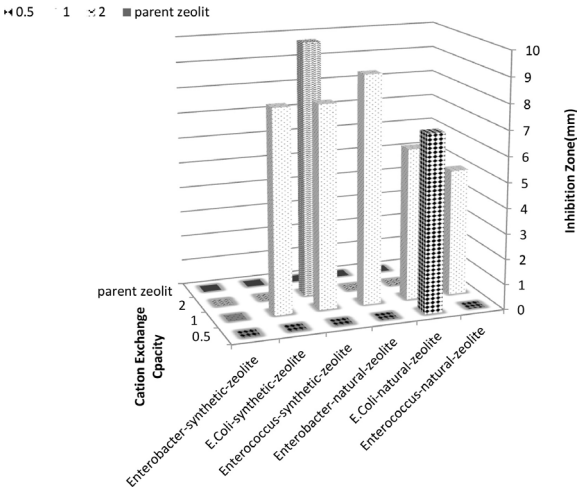

Fig. 5. Comparing the antibacterial activity of CPC-zeolites (natural and synthetic) in the different CEC (0.5 CEC: 0.02 g, 1.00 CEC: 0.04 g, and 2.00 CEC: 0.08 g) against *E. coli*, *Enterobacter*, and *Enterococcus* bacteria.

3.3. Microbroth dilution method

As was reported in Tables 2 and 3, the use of modified zeolite against three examined bacteria shows a remarkable result. The microdilution method was used to determine the MIC values and antimicrobial potential of the modified zeolite

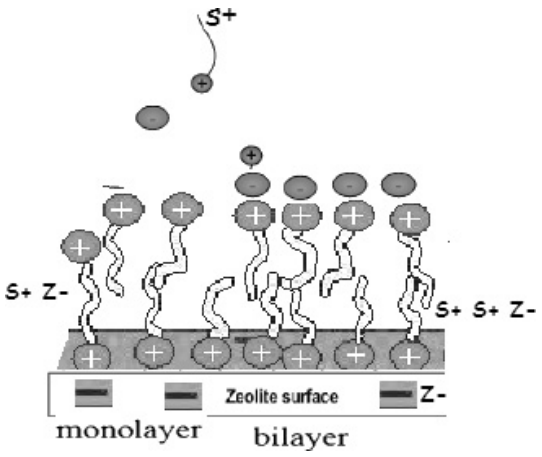

Fig. 6. Schematic representation of the adsorption surfactant onto the zeolite surface. The following parameters were used: Z–, anion; S+, free surfactant molecule; S+ Z–, surfactant adsorbed in the monolayer; and S+ S+ Z–, surfactant adsorbed in the bilayer.

Table 2
Minimum inhibitory concentration and minimum bactericidal concentration (mM) of CTAB-zeolite against standard screening strains of bacteria

Zeolite type	Type of bacteria	CTAB concentration according to CEC[a]	Optical density	CFU ($\times 10^8$/mL)
Natural	*E. coli*	0.5	0.37	<1.5
		1	0.44	<1.5
		2	0.649	>3
	Enterococcus	0.5	0	0
		1	0	0
		2	0	0
	Enterobacter	0.5	0	0
		1	0	0
		2	0	0
Synthetic	*E. coli*	0.5	0.67	>3
		1	0.57	3
		2	0.49	≤3
	Enterococcus	0.5	0.069	≅0
		1	0.086	≅0
		2	0.021	≅0
	Enterobacter	0.5	0	0
		1	0	0
		2	0	0
	+ve[b]		0.02	0
	–ve[c]		0.123	1.5

[a]CEC, cation exchange capacity.
[b]Positive control (+ve).
[c]Negative control (–ve).

Table 3
Minimum inhibitory concentration and minimum bactericidal concentration (mM) of CPC-zeolite against standard screening strains of bacteria

Zeolite type	Type of bacteria	CTAB concentration according to CEC[a]	Optical density	CFU ($\times 10^8$/mL)
Natural	*E. coli*	0.5	0.80	>3
		1	0.58	3
		2	0.50	>3
	Enterococcus	0.5	0	0
		1	0	0
		2	0	0
	Enterobacter	0.5	0	0
		1	0.043	≅0
		2	0	0
Synthetic	*E. coli*	0.5	0.46	<3
		1	0.59	3
		2	0.67	>3
	Enterococcus	0.5	0.084	≅0
		1	0.052	≅0
		2	0.039	≅0
	Enterobacter	0.5	0.319	<1.5
		1	0	0
		2	0	0
	+ve[b]		0.02	0
	–ve[c]		0.123	1.5

[a]CEC, cation exchange capacity.
[b]Positive control (+ve).
[c]Negative control (–ve).

Fig. 7. MIC method culture without adding (A) and with adding surfactant-zeolite (B).

[60]. The OD of tested tubes contain each concentration of the modified zeolite determined at 625 nm and were compared with McFarland turbidity standards (Fig. 7) [61]. According to the obtained result, natural and synthetic in both modified zeolites (0.5, 1.00, and 2.00 CEC) were the most effective compound having the minimum OD value with widest spectrum of antibacterial activity on the *Enterococcus* and *Enterobacter* as compared with *E. coli*. The result shows that CTAB-zeolite prevents bacterial growth [62]. On the other hand, the result of the MIC test confirms disc diffusion method. Because in both methods, modified zeolite with CTAB and CPC have an excellent effect on *Enterobacter* and *Enterococcus*.

4. Conclusion

This study has environmental applications, as it shows antimicrobial behavior of cationic-modified zeolite, which will be the basis for future research on developing alternative new disinfectants in water and wastewater treatment processes. The results of FTIR support successful modification of zeolites by surfactants. The results show that zeolite modified by surfactants improves the antibacterial characteristic of zeolites. The results revealed that electrostatic forces were the dominant mechanism in the antibacterial activity of the modified zeolites. The result of this study shows that modified zeolite is effective on the Gram-positive bacteria more than Gram-negative bacteria. In summary, zeolite is a natural and harmless substance, which can be used to support surfactants in disinfecting water and wastewater. Therefore, further study is needed on the applicability and cost-effectiveness of applying this material as a disinfectant in the water and wastewater treatment process.

Acknowledgments

The authors thank the Health Sciences Research Center, Student Research Committee, and the Mazandaran University of Medical Sciences (Grant number: 96.15 and 96.16).

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