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Antibacterial activity of amine-functionalized silver-loaded natural zeolite clinoptilolite

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ABSTRACT

The antibacterial activity of amine-functionalized silver-loaded natural zeolite clinoptilolite was analysed against Gram-negative *Escherichia coli* ATCC 11229 and a comparison was made with raw clinoptilolite, silver-loaded clinoptilolite, and amine-functionalized clinoptilolite with APTES (3-Aminopropyl) triethoxysilane. Results from the characterization of the samples using Fourier transform infrared (FTIR) spectroscopy showed that the silver-loaded clinoptilolite was functionalized with APTES without affecting the original framework structure of the clinoptilolite. The antibacterial activity of the analysed samples based on disc diffusion technique (DDT) and minimum inhibition concentration (MIC) showed that the silver-loaded clinoptilolite (150 % of cation exchange capacity of clinoptilolite) had the highest antibacterial activity, as compared to amine-functionalized silver-loaded zeolite. This study showed that the functionalization of silver-loaded clinoptilolite could affect the release and action of silver ions to kill or inhibit bacterial growth. However, it showed that the natural zeolite clinoptilolite could become a good carrier system for the antibacterial metal ion, especially silver ions.

Keywords: *Zeolite, clinoptilolite, antibacterial agent, silver, amine-functionalization*

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INTRODUCTION

Problems and issues with bacterial resistance to antibiotics have driven the usage of other alternative antibacterial agents such as metal ions, particularly silver ions, as a powerful antibacterial agent for household products [1,2]. However, the release of high amounts of metal ions into the environment creates secondary problems, including environmental pollution that needs to be tackled effectively. Furthermore, a higher amount of metals in the aquatic system than permissible can lead to health hazards, causing water-borne diseases [3]. Therefore, one alternative approach is by immobilization of silver ions by a carrier system to control the release of this metal ion.

There are various carrier systems for immobilizing antibacterial agents. The effective examples of a carrier system are kaolinite and zeolites, which are examples of inorganic minerals composed of negatively charged aluminosilicate framework structure that possesses high cation exchange capacity (CEC) and stability. Because of this characteristic, these minerals were used as a carrier system for not only a single antibacterial agent but also two or more antibacterial agents and compounds, creating hybrid organic-inorganic antibacterial agents. Examples of hybrid organic-inorganic antibacterial agents are natural kaolinite-loaded chlorhexidine and zinc ions [4], amine-functionalized silver-loaded zeolite Y [5,6], and natural zeolites for silver ions and surfactants [7]. Previous reports showed that the attachment of organic compounds (i.e. chlorhexidine, surfactant) on metal-loaded kaolinite and zeolites improved the antibacterial activity because there are synergistic effects between organic and inorganic antibacterial agents in a carrier system [4-7]. Furthermore, this could also reduce the number of used metal ions acting as an antibacterial agent.

Therefore, this study aimed to prepare functionalized silver loaded natural zeolite clinoptilolite with amine ((3-Aminopropyl) triethoxysilane or APTES) and analyse its antibacterial activity. Clinoptilolite was selected in this study as a carrier system because it had high CEC (Cation Exchange Capacity) and abundance. However, it had some disadvantages, including containing impurities such as other phases, and heterogenous surface structure and morphology as compared to synthetic zeolites. It was hypothesized that the natural zeolite clinoptilolite could adsorb and retain silver ions inside the framework; and the silver-loaded clinoptilolite could be functionalized with APTES, which could be similar to our previous works in which silver-loaded zeolite Y showed an enhanced antibacterial activity after being functionalized with APTES [5, 6]. Furthermore, there have been no studies reported on amine-functionalized, silver-exchanged natural clinoptilolite as an antibacterial agent. This study would potentially be giving insights on the importance of functionalized zeolite in enhancing the antibacterial activity of raw and modified zeolites.

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EXPERIMENTAL

Samples preparation

Natural zeolite clinoptilolite (Cli) was taken from Indonesia and supplied by Provet Group of Companies Sdn Bhd. The Cli sample contained clinoptilolite as the dominant species and quartz as the minor impurity [8]. The CEC of the Cli determined were based on the Na^+ saturated technique by Ming and Dixon [9] which was 175 meq/100 g. Table 1 shows the list of samples that have been prepared in this study.

Table 1: List of samples and their description

No.	Sample Name	Description
1.	Cli	Clinoptilolite
2.	CS40	Clinoptilolite + Ag (40 CEC % of Cli)
3.	CS150	Clinoptilolite + Ag (150 CEC % of Cli)
4.	CA5	Clinoptilolite + APTES (5 %)
5.	CA10	Clinoptilolite + APTES (10 %)
6.	CS40-A5	CS40 + APTES (5 %)
7.	CS40-A10	CS40 + APTES (10 %)
8.	CS150-A5	CS150 + APTES (5 %)
9.	Cs150-A10	CS150 + APTES (10 %)

The Ag-loaded clinoptilolite (CS40 and CS150) was prepared by loading Ag on Cli that satisfied the 40 % and 150 % of the CEC of the Cli. For the preparation of CS40 and CS150, the $AgNO_3$ (Merck) was weighed (0.713 and 2.676 g, respectively) and dissolved in 500 mL of deionised water. Then, 6.0 g Cli was added into the $AgNO_3$ solution. The suspension was stirred in dark conditions for 16 hours. The suspension was then filtered using a filter paper (Macherey-Nagel) to separate the solid and liquid portions. Finally, the solid portion was dried in an oven at 80 °C overnight. The dried solid was crushed with mortar and pestle, and sieved to obtain homogenous sample powder particles. The functionalized Cli with APTES was obtained by reacting 5 % and 10 % APTES (Aldrich) with Cli producing CA5 and CA10, each respectively [5]. Initially, 3 mL and 6 mL of APTES were added into two sets of beakers containing 4.0 g Cli in 60 mL dichloromethane (QRec) for the preparation of CA5 and CA10, respectively. On the other hand, the functionalized Ag-Cli samples were prepared by loading APTES (5 % and 10 %)

on Ag-Cli (CS40 and CS150). And the rest of the procedures were similar to that of the preparation of functionalized Cli (CA5 and CA10). After the suspension was prepared, the rest of the procedure was similar to that of the preparation Ag-Cli until the powdered samples for each sample were obtained.

Characterization technique

The samples were characterized by using Fourier transform infrared (FTIR) spectroscopic instrument (Thermo Scientific Nicolet IS5-IR spectrometer) with the Attenuated Total Reflection (ATR) technique. The FTIR spectra was recorded in the range of 4000 to 500 cm^{-1} with 4 cm^{-1} wavenumber resolution.

Antibacterial assays

Two antibacterial assays were used for the analysis of antibacterial activities of the samples: Gram-negative *Escherichia coli* ATCC (American Type Culture Collection) 11229: (1) Disc diffusion technique (DDT) and (2) minimum inhibition concentration (MIC). For DDT, all samples (0.10 g) were pressed into a disc (1.30 cm) using an E-Z press at 4000 tonnes. A colony of the bacteria was suspended in saline solution (0.9%) and shaken in an incubator shaker (200 rpm, 37°C). The suspension was adjusted according to a 0.5 McFarland standard (1.5×10^8 CFU (Colony Forming Unit)/mL) by visual comparison between the turbidity of the suspension with that of the McFarland standard. The bacterial suspension was then spread on a Muller Hinton Agar (MHA) evenly using a sterilized cotton bud, and the sample disc was subsequently placed on top of the bacteria surface. The plates were then incubated at 37 °C for 24 h. The clear zone was observed and measured after 24 h.

The determination of MIC started with the preparation of bacteria suspension in Luria Bertani (LB) broth. A colony of the bacteria was inoculated in 100 mL LB broth and incubated overnight at 37 °C by shaking at 200 rpm. Then, 10 mL of the bacterial suspension was added into 100 mL of fresh LB broth and incubated until the OD reached between 0.6 and 0.8 at 550 nm. The suspension was centrifuged and washed several times with saline solution. The bacteria in saline solution were then added with the powdered samples at different solid to liquid ratio (0.3 to 12 g/L). The mixture of sample and bacteria was shaken at 37 °C for 30 min at 100 rpm. Finally, 10 μL of the bacterial suspension was dropped onto a nutrient agar plate based on the dropped plate technique. The plate was incubated at 37 °C overnight and the bacterial growth was observed. The experiment was done in triplicates. The MIC value was determined based on the lowest ratio or concentration (solid/liquid) that could inhibit bacterial growth.

RESULTS AND DISCUSSION

Characterization by FTIR technique

The characterization of the samples Cli, Ag-loaded Cli, amine-functionalized Cli, and amine-functionalized Ag-Cli using FTIR spectroscopic technique provided the information on two important findings: (1) structural stability, and (2) successful functionalization of the zeolite [5]. Figure 1 shows the FTIR spectra of the samples in the range 4000 to 500 cm^{-1}.

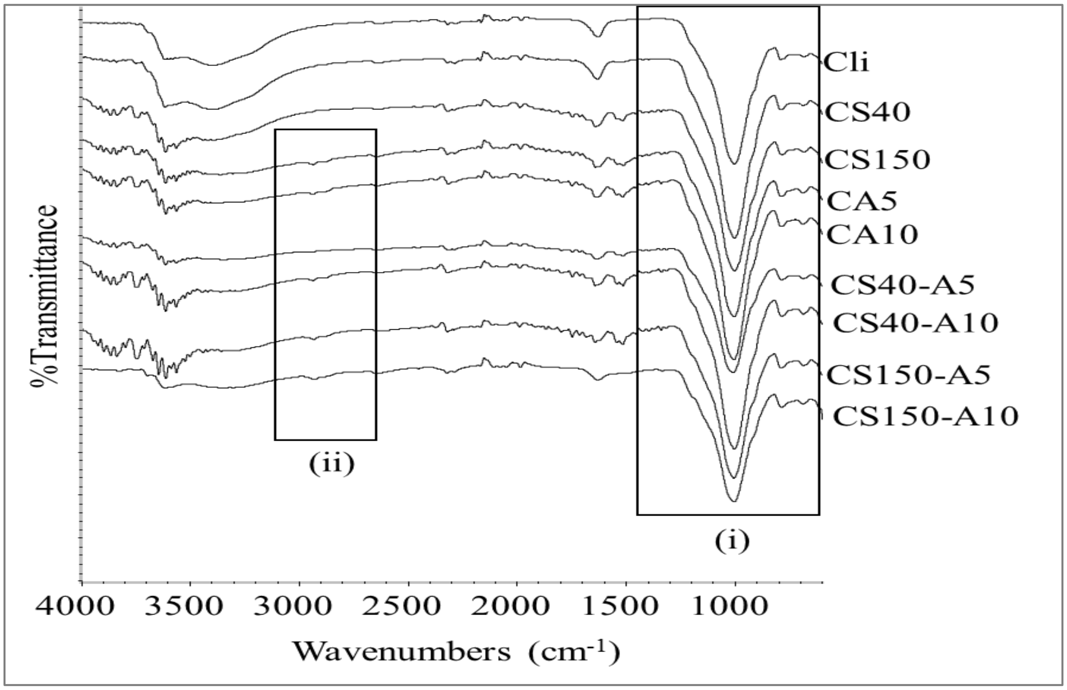

Figure 1: FTIR spectra of raw zeolite (Cli), Ag-loaded Cli, amine-functionalized Cli and amine-functionalized Ag-Cli (Sample names can be referred to Table 1) with two regions ((i) 1200-500 cm^{-1} and (ii) 3000-2800 cm^{-1})

The FTIR spectrum of the Cli at region (i) showed three bands that were specific for zeolite type clinoptilolite. At around 1000 cm^{-1} (strong band), representing asymmetric stretching of Si-O-Si and Si-O-Al, and at 800 cm^{-1} (medium band) and 600 cm^{-1} (medium band) representing symmetric stretching of Si-O-Si and Si-O-Al [10]. The FTIR spectra for all modified samples had similar bands at this region proving that there were no structural changes after the Cli had been loaded with Ag and functionalized with APTES. This was due to the modification process involving ion exchange and surface structure without distorting the main structure of the zeolite.

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The Ag^+ ions from $AgNO_3$ were exchanged with cations that neutralized the negatively charged framework structure of the Cli. The Ag^+ in Ag-Cli samples resided inside the structure without affecting the aluminosilicate structure of the zeolite [11].

Besides that, there were some additional small and weak bands for functionalized Cli and Ag-Cli at region (ii), between 2800 and 2900 cm^{-1}. These two additional bands indicated the presence of APTES on the Cli surfaces. The former band represented symmetric C-H whereas the latter band represented asymmetric C-H for APTES molecules [5,12]. These bands were small and weak because of the lower amount of APTES on the zeolite surface, as well as higher bonding energy of Si-O-Al and Si-O-Si made the zeolite framework as compared to C-H in APTES. From these FTIR spectra, it was proven that the zeolite clinoptilolite had successfully loaded with Ag and subsequently functionalized with APTES without damaging the main structure of the zeolite. The zeolite Cli acted as a carrier system for Ag and APTES.

Antibacterial activity

The antibacterial activity of the amine-functionalized Ag-Cli was analysed based on DDT and MIC, using a few control samples which were unmodified Cli, Ag-Cli, and amine-functionalized Cli. DDT and MIC were used for qualitative and quantitative antibacterial analyses, respectively. In addition to that, the DDT referred to solid to solid interface, whereas MIC referred to solid to liquid interface. Table 2 shows the inhibition zone values and images from DDT, MIC values (g/L), and the images from the MIC technique.

The Cli sample did not have antibacterial activity because there were no antibacterial agents in the Cli structure and this result was similar to other previous papers [5, 13]. However, all modified Cli showed antibacterial activity especially in the results based on the DDT. The trend for antibacterial activity for the overall samples based on DDT was as follows: CS150 > CS40 > CS40-A5/CS150-A5 > CS150-A10 > CS40-A10 > CA5 > CA10. The trend based on MIC was as follows: CS150 > CS40/CS150-A5 > CS40-A10/CS150-A10 > CS40-A5 > CA5/CA10. There were different trends between the results from DDT and MIC probably due to the different interface conditions in both techniques where the DDT was based on solid to solid interface and MIC was based on solid to liquid interface. These different interfaces resulted in a different antibacterial mechanism that was largely contributed by the interactions between the bacteria and antibacterial agents inside the zeolite framework. In addition to that, there was no correlation between the percentage of APTES attached to the zeolite (5 and 10 %) and the antibacterial activity of the amine-functionalized Cli.

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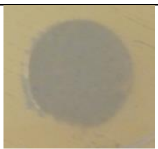
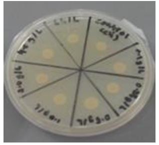
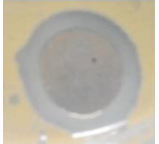
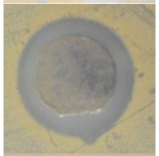
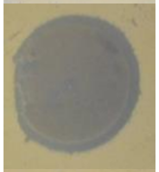
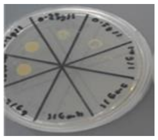
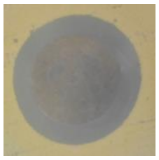
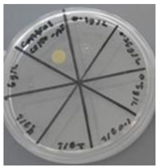
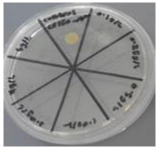
Table 2: Antibacterial activities of the samples based on DDT and MIC values.

No	Sample Name	Inhibition zone (cm)	DDT Images	MIC value (g/L)	Image from dropped plate
1.	Cli	0.00		> 6.00	
2.	CS40	2.07		0.25	
3.	CS150	2.13		0.1	
4.	CA5	1.53		> 6.00	
5.	CA10	1.47		> 6.00	
6.	CS40-A5	1.93		2.00	
7.	CS40-A10	1.87		0.50	
8.	CS150-A5	1.93		0.25	
9.	CS150-A10	1.90		0.50	

From the trend, CS150 with a high amount of Ag-loaded clinoptilolite (150% of the CEC of the Cli) showed the highest antibacterial activity. Thus proving that the antibacterial activity of the modified zeolite was contributed by the Ag [14]. Furthermore, this sample (CS150) had higher antibacterial activity than CS40 which had a lower Ag amount loaded Cli. On the other hand, the amine-functionalized Ag-Cli (CS150-A5 and CS150-A10) reduced the antibacterial activity of the Ag-Cli (CS150). This might be due to the lower Ag amount in the zeolite because of the release of Ag ions from the zeolite framework during the functionalization process or the APTES on the zeolite surfaces had blocked the release of Ag ions into the solution, preventing the antibacterial actions by the Ag. This phenomenon also occurred for sample Ag-Cli with a lower amount of Ag (CS40 as compared to CS40-A5 and CS40-A10). This result was different compared to a previous study by Hanim *et al*. [5] in which the functionalization of silver-loaded zeolite NaY with APTES enhanced the antibacterial activity of silver-loaded zeolite NaY. Zeolite NaY (CBV100) is a synthetic zeolite that has homogenous particles, sodium ion (Na^+) as its cations, high crystallinity, and low impurities. Whereas natural zeolite clinoptilolite has a heterogeneous structure, various cations, lower crystallinity, and high impurities. The difference in terms of these characteristics might contribute to the different effect of functionalization of natural zeolite Cli, eventually affecting the antibacterial mechanisms.

CONCLUSION

Amine-functionalized Ag-loaded clinoptilolite was prepared and characterized using FTIR, and its antibacterial activity was compared with raw clinoptilolite; Ag-loaded clinoptilolite, and amine-functionalized clinoptilolite. The antibacterial activity against Gram-negative *E. coli* of amine-functionalized Ag clinoptilolite was lower than the Ag loaded clinoptilolite might be due to APTES preventing the antibacterial action of Ag ions. Hence, the natural zeolite clinoptilolite could be used as a carrier system for organic and inorganic antibacterial agents.

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