Biofilm inhibition and antimicrobial properties of silver ion-exchanged zeolite A against *Vibrio* spp marine pathogens

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This study highlights a potential application of ion-exchanged Zeolite A against marine microbial pathogens and their biofilms.

Abstract: A challenging problem in the aquaculture industry is bacterial disease outbreaks which results in the global reduction of fish supply and foodborne outbreaks. Biofilms in marine pathogens protect against antimicrobial treatment and host immune defense. Zeolites are minerals of volcanic origin made from crystalline aluminosilicates which are useful in agriculture and in environmental management. In this study, silver ion-exchanged zeolite A of four concentrations; 0.25M (AgZ¹), 0.50M (AgZ²), 1.00M (AgZ³) and 1.50M (AgZ⁴) were investigated for biofilm inhibition and antimicrobial properties against two predominant marine pathogens *V. campbellii* and *V. parahemolyticus* by employing the Minimum Inhibitory Concentration (MIC), Crystal Violet Biofilm Quantification assays as well as Scanning Electron Microscopy. In the first instance, all zeolite samples AgZ¹-AgZ⁴ showed antimicrobial activity for both pathogens. For *V. campbellii* AgZ⁴ exhibited the highest MIC at 125.00 µg/ml while for *V. parahaemolyticus* the highest MIC was observed for AgZ³ at 62.50 µg/ml. At sublethal concentration, biofilm inhibition of *V. campbelli* and *V. parahemolyticus* by AgZ⁴ were observed at 60.2% and 77.3% inhibition respectively. Scanning electron microscopy exhibited profound structural alteration of the biofilm matrix by AgZ⁴. This is the first known study that highlights the potential application of ion-exchanged Zeolite A against marine pathogens and their biofilms.

Keywords: Biofilms, Zeolite A, *Vibrio* sp, Antimicrobials

1. Introduction

Aquaculture farming has been the fastest growing food producing sector in the last few decades and an important industry in many developing countries. However, the industry currently faces a threatening challenge due to the bacterial disease outbreaks resulting in high mortality rates in the aquaculture population (1,2). This is in part due to extensive use of antibiotics in fish farms leading to antimicrobial resistance in fish pathogens (3-5). Vibriosis is an important bacterial disease in wild and farmed marine fishes which results in severe economic loss of more than USD 1 billion (6). In addition, bacteria from seafood sources have been associated with foodborne outbreaks (7). Biofilms are self-assembled communities of microorganisms embedded in a self-developed polymeric matrix to a biotic or abiotic surface and comprise exopolysaccharides, proteins, and extracellular DNA (eDNA) (8). Biofilms shield bacteria from external disturbances for improved resilience to
the environmental conditions as well as enhancing virulence. They primarily confer protection against antimicrobial agents, disinfectants and host defense mechanisms, making them more difficult and expensive to treat (9). The prevalent challenge has necessitated efforts on new antibacterial materials that can effectively inhibit the growth of the resistance aquatic pathogens while minimizing negative impacts on human, animal health and to the environment. Recently, several studies on natural therapeutics from plants and immunostimulants (10-12), and by alternative inorganic material have been explored (13-14), including the application of zeolites as antimicrobial agents (15-17).

This study aimed to investigate antimicrobial and antibiofilm applications of silver ion-exchanged zeolite A against marine bacterial pathogens *V. parahaemolyticus* and *V. campbelli*. Study findings strongly indicated antimicrobial and antibiofilm characteristics of the silver ion-exchanged zeolite A against the bacterial pathogens, with the highest MIC levels observed for AgZ4 (1.50M) for *V. campbelli* and AgZ3 (1.00M) for *V. parahaemolyticus*. Scanning electron microscopy exhibited profound breakages in the biofilm structures of both marine pathogens when grown in media added with 1.50M silver ion-exchanged zeolite A (AgZ4). Taken together, this study highlights the potential application of ion-exchanged zeolite A against marine pathogens and their biofilms.

2. Materials and Methods

**Liquid Ion Exchange of Zeolite A**

The initial synthesis of zeolite A from bentonite clay comprised the activation of precursor bentonite clay by thermochemical treatments in HCl, addition of alkaline activators, aging and crystallization processes as well as characterization by X-Ray diffraction (XRD) and Scanning Electron Microscopy (SEM) analysis (data published elsewhere).

For liquid ion exchange, the integration of silver (Ag) ion into zeolite A was performed by using the liquid ion exchange method as described by Demirci *et al.*, (17) with slight modifications. Briefly, 1 g zeolite A was added into 10 ml of four AgNO₃ concentrations: 0.25 M, 0.50 M, 1.00 M and 1.50 M (denoted AgZ1, AgZ2, AgZ3 and AgZ4 respectively) and mixed at 150 rpm for 3 days. The obtained zeolite A were then vacuum filtered, washed with deionized water and dried at 80°C overnight.

In order for confirm the capacity of ion exchange in the AgZ, ICP-OES analysis was carried out. Briefly, 200 mg of AgZ1, AgZ2, AgZ3 and AgZ4 was added into separate tubes containing 14 ml of acid mixture, aqua regia (40% HNO₃ + 60% HCl) and left overnight at room temperature. 4 ml of aqua regia solution was then added into the tubes and mixed for 30 minutes at 80°C before being filtered and diluted prior the ICP-OES analysis.

**Minimum Inhibition Concentration (MIC) Assay of Ag-exchanged zeolite A**

The Minimal Inhibitory Concentration (MIC) assay of Ag-exchanged zeolite A against *V. parahaemolyticus* and *V. campbellii* was carried out as described (18). Briefly, frozen stocks of *V. campbellii* and *V. parahaemolyticus* were grown on Nutrient Agar (NA) supplemented with 2% of NaCl and incubated overnight at 37°C. Overnight cultures were inoculated into Tryptone Soy Broth (TSB) and absorbances were adjusted accordingly to an initial starting OD₆₀₀ of 0.05. As seen in Table 1, samples AgZ1, AgZ2, AgZ3 and AgZ4 of respective AgZ concentrations were then added.

Table 1 Concentrations of Silver Ion Zeolites used for the Minimum Inhibitory Concentration Assay.
For each bacterial species, doubling dilutions up to 1/512 of AgZ1, AgZ2, AgZ3 and AgZ4 were firstly carried out. 500 µl of liquid inoculum (OD$_{600}$ adjusted to 0.05) was then added and incubated in a shaking 37°C incubator for 16 hours. 100 µl of the culture was then plated and spread on to Nutrient Agar (NA) plates supplemented with 2% NaCl and further incubated overnight at 37°C with appropriate controls. The minimum inhibitory concentration (MIC) of AgZ1, AgZ2, AgZ3 and AgZ4 was determined by the lowest concentration of samples that did not exhibit bacterial growth.

Quantitation of Bacterial Biofilm Density by Crystal Violet Assay

The biofilm density of AgZ treated biofilm were quantitated by the crystal violet assay using the methods described by O’Toole (19) with modifications. Briefly, cultures of V. campbellii and V. parahaemolyticus were incubated overnight at 37°C in Tryptone Soy Broth (TSB) supplemented with 2% NaCl.

For the biofilm assay, the sublethal concentration obtained from the MIC assay from was selected as the starting assay concentration. Briefly, doubling dilutions of the sample were performed in a 96 well microtiter plate (Corning) containing Tryptone Soy Broth supplemented with 2% NaCl. 50 µl of the overnight cultures (with OD$_{600}$ adjusted to 0.05) were then inoculated into each well and was then incubated at 37°C for 72 hours. Following incubation, wells were washed three times with distilled H$_2$O, desiccated at 50°C, stained with 1% crystal violet for 30 min and added with 95% ethanol. The absorbance of solubilized dye was then determined at 570nm (Shimadzu Spectramax).

Scanning Electron Microscopy (SEM) of V. campbellii and V. parahaemolyticus biofilms

The cell morphologies of V. campbellii and V. parahaemolyticus biofilms grown in TSB media and in TSB with AgZ4 was analyzed by SEM. Briefly, 5 ml overnight liquid cultures of V. campbellii and V. parahaemolyticus were cell fixated according to protocol by Gomes and Mergulhão (20) with modifications. The biofilm samples were then fixed in 5% glutaraldehyde prepared in 0.1 M PBS pH 7.2 at 4°C for 12 hours. Following the fixation process, the samples were dehydrated by introduction into a series of ethanol solution of varying concentration gradients (35%, 50%, 75%, 95%, and 2 x 100%). The dehydrated samples were then immersed in HMDS for 10 minutes. Upon dehydration the samples were dried overnight and then sputter–coated with platinum before being analyzed by the SEM (Hitachi SEM, S 3400N).

3. Results

The incorporation of silver ion (Ag) into the zeolite A framework (AgZ) was determined by introducing the zeolite sample to four different concentrations of Ag solution AgZ1, AgZ2, AgZ3 and AgZ4. During this process, the potassium and sodium ions that exist in the zeolite framework was exchanged by metal ions. ICP-OES analysis of AgZ1, AgZ2, AgZ3 and AgZ4 are as shown in Table 2.

<table>
<thead>
<tr>
<th>Metal-Ion (Zeolite/Concentration(M))</th>
<th>0.25 M</th>
<th>0.50 M</th>
<th>1.00 M</th>
<th>1.50 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver (AgZ)</td>
<td>AgZ1</td>
<td>AgZ2</td>
<td>AgZ3</td>
<td>AgZ4</td>
</tr>
</tbody>
</table>
Table 2 Silver ion content (mg/g) of zeolite of samples

<table>
<thead>
<tr>
<th>Metal Ion/Concentration</th>
<th>0.25 M (1)</th>
<th>0.50 M (2)</th>
<th>1.00 M (3)</th>
<th>1.50 M (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver (AgZ)</td>
<td>80.949 ± 0.389</td>
<td>160.041 ± 1.328</td>
<td>177.481 ± 1.483</td>
<td>190.511 ± 1.989</td>
</tr>
</tbody>
</table>

As seen in Table 2, the highest silver ion content was observed for AgZ4 at 190.511 ± 1.989 mg/g, followed by the lowest concentration for AgZ1 at 80.949 ± 0.389 mg/g.

Minimum Inhibitory Concentration (MIC) of *V. campbellii* and *V. parahaemolyticus* in AgZ

The MIC values for each zeolite sample against *V. campbellii* and *V. parahaemolyticus* are as summarized in Table 3 below.

<table>
<thead>
<tr>
<th>Bacterial Pathogens</th>
<th>MICs value of four different Silver Ion Loaded Zeolite A (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AgZ1</td>
</tr>
<tr>
<td><em>V. campbellii</em></td>
<td>2.0000</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>0.5000</td>
</tr>
</tbody>
</table>

As seen in Table 3, both *V. campbellii* and *V. parahaemolyticus* showed susceptibility to all four AgZ1-AgZ4 concentrations. For *V. campbellii*, the highest MIC was observed in AgZ4 at 0.1250 mg/ml. For *V. parahaemolyticus*, the highest MIC was observed in AgZ3 at 0.0625 mg/ml. The lowest MIC for both *V. campbellii* and *V. parahaemolyticus* was observed in AgZ1 at 2.0 mg/ml and 0.5 mg/ml respectively.

Quantitation of Bacterial Biofilm Density by Crystal Violet Assay

The AgZ4 MIC concentration for both pathogens were further selected for biofilm inhibition crystal violet assay. Figure 1 demonstrated biofilm inhibition percentages of ion-exchanged zeolite in comparison with the untreated control. As shown in Figure 1, at 570nm absorbance, biofilm formation of AgZ4 against *V. campbellii* and *V. parahaemolyticus* exhibited up to 60.2% and 77.3% inhibition respectively.
Figure 1. Biofilm Inhibition of *V. campbellii* and *V. parahaemolyticus* by 1.50M AgZ4 assessed by CV staining.

### Biofilm Inhibition (%) of 1.50M AgZ4 against *V. campbellii* and *V. parahaemolyticus*

<table>
<thead>
<tr>
<th>Biofilm Inhibition (%)</th>
<th><em>V. campbellii</em></th>
<th><em>V. parahaemolyticus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>60.2</td>
<td></td>
<td>77.3</td>
</tr>
</tbody>
</table>

Figure 1. Biofilm Inhibition of *V. campbellii* and *V. parahaemolyticus* by 1.50M AgZ4 assessed by CV staining. Biofilm inhibition of *V. campbellii* and *V. parahaemolyticus* isolates after 24 h growth with 1.50M AgZ4, was assayed by CV staining.[57%]

**Scanning Electron Microscopy of *V. campbellii* and *V. parahaemolyticus* in AgZ4**

Figure 2(a) and 3(a) represent the SEM images of untreated samples of *V. campbellii* and *V. parahaemolyticus* respectively, while Figure 2(b) and 3(b) represent the SEM images of *V. campbellii* and *V. parahaemolyticus* after growth in media culture treated with 1.50M AgZ4.

Figure 2 (a) shows clustering and aggregation of *V. campbellii* covered with a network of extracellular matrix (ECM) as a typical representation of intact biofilms. In contrast, Figure 2 (b) showed markedly reduced ECM and aggregation of bacteria.

**Figure 2.** SEM Analysis of untreated *V. campbellii* biofilm formation (a), and treated with AgZ4 (b).

Figure 2. SEM Analysis showing (a) untreated *V. campbellii* biofilm bacteria embedded in extracellular matrix biofilm and (b) *V. campbellii* treated with AgZ4 where profound loss of biofilm matrix is seen.
Similarly, Figure 3 (a) below showed clustering and aggregation of untreated *V. parahaemolyticus* covered with a fine network of extracellular matrix (ECM). In contrast, as seen in Figure 3 (b) *V. parahaemolyticus* exposure to AgZ4 showed a markedly reduced ECM and cell clustering.

**Figure 3.** SEM Analysis of untreated *V. parahaemolyticus* biofilm formation (a), and treated with AgZ (b).

**Figure 3: SEM Analysis showing (a) untreated *V. parahaemolyticus* biofilm bacteria embedded in extracellular matrix biofilm and (b) *V. parahaemolyticus* treated with AgZ4 where profound loss of biofilm matrix is seen.**

4. Discussion

*V. parahaemolyticus* is a prevalent food-poisoning bacterium associated with seafood consumption, typically causing self-limiting gastroenteritis and commonly found in temperate and tropical marine and coastal waters globally (21). Vibriosis is a systemic bacterial infection in farmed and wild marine fishes, which is considered to be a profoundly significant problem due to intensive economic losses in aquaculture industry worldwide (22). *V. campbellii* is as an emerging marine pathogen recently associated with diseased farm shrimps (23). The development of biofilms in marine bacteria further complicate bacterial disease management in aquaculture as the extracellular matrix in biofilms confer protection against antimicrobial substances (24). Zeolites have been found to be useful in various applications in aquaculture especially in the purification of hatchery tanks (25). This study aimed to investigate antimicrobial and antibiofilm applications of silver ion-exchanged zeolite A against marine bacterial pathogens *V. parahaemolyticus* and *V. campbellii*. The applicability of the bentonite-based silver ion loaded zeolite A for samples AgZ1, AgZ2, AgZ3 and AgZ4 as antibacterial agents was evaluated against *V. campbellii* and *V. parahaemolyticus*.

The initial ICP-OES analysis of AgZ1, AgZ2, AgZ3 and AgZ4 revealed the successful incorporation of Ag ion into the zeolite samples to be typically in direct correlation with the increase in Ag ion concentration introduced. The highest Ag ion concentration was observed in sample AgZ4 (1.50M) at 190.511 ± 1.989 mg/g. At 0.50M, the Ag ion concentration of AgZ2 was double that of AgZ1 at 160.041 ± 1.328 mg/g compared to 80.949 ± 0.389 mg/g. Nonetheless doubling concentration increases observed for AgZ2 was not indicated in AgZ3 and AgZ4. A possible explanation for this is saturation of available sites in AgZ3 and AgZ4 for the ion exchange to occur.

In general, Minimum Inhibitory Concentration (MIC) evaluation against *V. campbellii* and *V. parahaemolyticus* firstly revealed that the incorporation of Ag ions into the zeolites
samples AgZ1, AgZ2, AgZ3 and AgZ4 was successful in inhibiting bacterial growth. Silver (Ag) has long been established in various studies as an effective antibacterial agent. Several studies have also demonstrated the efficiency of Ag exchanged zeolite against many microbial pathogens (26-27) and concur with the findings of this study. Additionally, a typically inverse relationship between sample concentration and MIC for AgZ1, AgZ2, AgZ3 and AgZ4 was also observed. As seen in Table 3, a lower MIC which signifies stronger antibacterial activity, was indicated for each bacterial type as the concentration of metal ion in the zeolite increased. Therefore, the higher the Ag ion loading in the zeolite, the lower the concentration of ion-loaded zeolite A was needed to inhibit the growth of the bacteria. There are many studies into the mechanism of microbial killing by metal ion. A possible mechanism involves the ability of the metal ion to attach to the bacteria membrane through electrostatic interaction and drastically alter the integrity of the bacterial membrane. Consequently, it promotes the formation of reactive oxygen species, (ROS) which will induce the oxidative stress to the bacteria cell resulting the oxidation of cellular component, DNA damage, mitochondria damage and disruption of the cell membrane which lead to the death of the bacteria (28).

MIC studies also revealed that between the two bacterial species, V. parahaemolyticus indicated higher susceptibility against Ag ion compared to V. campbellii as lower MICs were observed across AgZ1-AgZ4. Under antibiotic pressure, bacterial phenotypes such as susceptibility, resistance, tolerance and persistence differ from one bacteria to the other. In a review by Li et al (29), the efficacy of antimicrobials are influenced by many factors including bacterial status, host factors and antimicrobial concentrations.

A typical feature of bacterial biofilms is the extracellular matrix which provides protection and structure to the cell population within it (30). The CV assay is a useful tool for rapid and simple assessment of biofilm formation differences between bacteria, as it stains the extracellular matrix as well as the aggregated bacterial cells (O’Toole, 2011). The CV biofilm assays of V. campbelli and V. parahaemolyticus isolates grown in AgZ4 overnight showed biofilm growth were effectively inhibited in the presence of AgZ4. However, V. parahaemolyticus biofilms were indicated to be more susceptible against AgZ4 with a percentage inhibition of 77.3% compared to V. campbelli at 60.2%. This concurred with the MIC assays which also showed a higher susceptibility of V. parahaemolyticus when compared against V. campbelli and demonstrated significant attenuation of biofilm formation against V. campbelli and V. parahaemolyticus.

The ability of Ag ion-exchanged zeolite AgZ4 to disrupt biofilm development for V. campbelli and V. parahaemolyticus was further supported by SEM analysis. As seen in Figures 2 and 3, the exposure of AgZ4 to both V. campbelli and V. parahaemolyticus displayed significant structural alteration of biofilm phenotypes when compared to bacterial isolates grown in the absence of AgZ4 including profound loss of the biofilm extracellular matrix (ECM) as well as markedly reduced cell aggregation. While SEM analysis of untreated isolates showed tight aggregation of cells held together by ECM, isolates exposed to AgZ4 showed higher numbers of singular isolates with lessened clustering. Breakages on the extracellular matrices of biofilms will result in increased susceptibility of bacteria against antibacterial agents and chemicals (31). Several studies have demonstrated biofilm inhibition by various antibiofilm agents including plant species (31-32). Despite much literature on antibiofilm activities of bacteria by zeolite, the mechanism of toxicity of AgZ against biofilms of V. campbelli and V. parahaemolyticus are still poorly understood.
Therefore future studies on the probability of modification of gene expression in the *Vibrio* polysaccharide (VPS) and matrix protein biosynthesis are recommended to further inform on genes or protein that are significantly affected by metal loaded zeolites.

5. Conclusions

Taken together, the results of this study strongly indicate the strong antibacterial and antifilm potentials of Ag-ion exchanged zeolite A which can be applied in the aquaculture industry to combat against infectious pathogens in particular *V. campbellii* and *V. parahaemolyticus*.

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**References**


hydrophobicity and quorum sensing among Vibrio parahaemolyticus isolates from food contact surfaces and the distribution of the genes involved in biofilm formation. *Biofouling*, 32(4), 497-509.


