Preparation of Hydroxyapatite-Titanium Dioxide Composite from Eggshell by Hydrothermal Method: Characterization and Antibacterial Activity

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Abstract: Hydroxyapatite (HA) has been widely used in biomedical applications. HA is prepared from natural sources of eggshell. The obtained HA is composited with TiO\(_2\) using the hydrothermal method at a temperature of 230 °C. The structure and morphology of HA-TiO\(_2\) composites are characterized by X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and a scanning electron microscope (SEM). Meanwhile, its antibacterial activity was tested on Staphylococcus aureus and Escherichia coli bacteria. The formation of the HA-TiO\(_2\) composite is evidenced by typical peaks on the XRD pattern for HA and TiO\(_2\). The FTIR spectrum shows that no bond formed between TiO\(_2\) and HA which indicates the formation of composites. The smallest crystallite size and the highest specific surface area were obtained from the composite with the composition of HA-TiO\(_2\) 30:70. In addition, the composition of the composite also shows the smallest particle size distribution. Therefore, the presence of TiO\(_2\) plays a significant role in determining the HA properties formed. Furthermore, the HA-TiO\(_2\) composite showed good antibacterial activity using disk diffusion and optical density (OD) methods. These results indicate that the synergistic combination of HA from eggshell with TiO\(_2\) has favorable properties for antibacterial activity.

Keywords: natural source; eggshells; hydroxyapatite; composites; titanium dioxide; antibacterial properties

1. Introduction

Hydroxyapatite (HA) is one of the inorganic compounds with the chemical formula Ca\(_{10}\)(PO\(_4\))\(_6\)(OH)\(_2\) [1,2]. HA has been widely used in biomedical applications, especially in bone and dental implants [3,4]. This is because HA is a component of bone minerals and a constituent of teeth in humans that has good bioactivity and biocompatibility [5,6]. HA is usually synthesized from chemicals in the form of H\(_2\)SO\(_4\) and Ca(OH)\(_2\), but in general, the process is quite complicated and the product lacks biocompatible properties [7]. The biocompatibility properties of the material for the purposes of biomedical application are conditions that must be met. Some researchers have modified the sources of synthesis materials used, as sourcing materials from nature is more often cheaper, easier to obtain, and most importantly demonstrates biocompatible properties [8]. HA from natural sources also contains ions such as cationic Na\(^+\), Zn\(^{2+}\), Mg\(^{2+}\), K\(^+\), Si\(^{2+}\), Ba\(^{2+}\), and anionic F\(^-\), Cl\(^-\), SiO\(_4^{2-}\), CO\(_3^{2-}\) [9,10]. Some sources of HA from natural materials are cow bones, blood clam shells, and fish bones [11]. Another study revealed the synthesis of HA from chicken eggshells [12]. Eggshells are the easiest natural source to obtain, are relatively inexpensive, and are easy to prepare [13,14]. Currently, many researchers are conducting research on
eggshells as an alternative treatment to replace bone damage in humans, since the eggshell itself contains a fairly high element of CaCO$_3$ which is useful as a biomaterial [15]. In addition, the eggshell produced in a year weighs 138,956 tons, and the CaCO$_3$ content of eggshells can reach 94% [16]. Thus, the chicken eggshell has a high potential for the synthesis of high-quality hydroxyapatite.

HA has the advantage that it can absorb bacteria by adsorbing molecules on its surface [17,18]. However, HA cannot decompose a molecule [19,20]. In addition, HA has properties that are resistant to ultraviolet radiation and X-rays. Thus, it is resistant to interference, especially to radiation [21,22]. To overcome the shortcomings, HA needs to be modified by adding other materials. Many studies have observed an increase in the effectiveness of HA due to the formation of composites with polymers [23], metals [24], or metal oxides [25]. The metal oxide that is commonly used is titanium dioxide (TiO$_2$), which is non-toxic, environmentally friendly, and has high mechanical stability, high photocatalytic activity, and antibacterial activity [26,27]. TiO$_2$ has the ability to deactivate Gram-positive and Gram-negative bacteria [28].

Based on the results of previous studies, the increase in the performance of HA composites is strongly influenced by the composition and modification of the structure as well as the synthesis method. Therefore, many researchers have prepared HA-TiO$_2$ composites by various methods such as sol-gel, solid state, and hydrothermal methods [29–31]. Ortiz et al. [32] synthesized TiO$_2$/HA composite by the sol-gel method followed by carbon dioxide supercritical drying. In this study, the precursors used were Ti(OBu)$_4$ and 2–5% mol HA. In addition, other studies showed that the addition of 15 and 30% TiO$_2$ to HA showed changes in the behavior of the hydroxyapatite matrix such as the crystallinity, morphology, and bioactivity [33,34]. In another study, HA-TiO$_2$ composite with the addition of TiO$_2$ by 25% can significantly increase the mechanical properties of HA [35,36].

In addition, several researchers have succeeded in synthesizing HA-TiO$_2$ composites to inactivate bacteria through photocatalytic degradation mechanisms [37]. By modifying HA with TiO$_2$, HA can adsorb molecules which will then be broken down by TiO$_2$. HA-TiO$_2$ composites with a ratio of 50–50 showed a bacterial decomposition of about 50% after 2 h of UV exposure [38,39]. In other research, the HA-TiO$_2$ composite with 60% TiO$_2$ showed good antibacterial activity [40]. Thus, the synthesis of HA and the formation of its composite with TiO$_2$ by the right composition can be used as an antibacterial material and is the focus of this study.

However, although several studies have succeeded in synthesizing various HA/TiO$_2$ composites [41], the degradation efficiency still needs to be improved because the research does not fully discuss the effect of compositional variations of the two materials. In addition, no reports have shown the synthesis of HA/TiO$_2$ composites from natural sources, especially from chicken egg shells. Therefore, in this study, we conducted the HA synthesis via a one-step hydrothermal method using the variant source of CaO from chicken eggshells [14]. The synthesized HA was then composited with various molar variations of TiO$_2$ by the hydrothermal method and observed for its antibacterial activity against Gram-positive and Gram-negative bacteria. The use of the hydrothermal method has the advantage that the synthesis results have high purity and crystallinity. In addition, the yield is more than 90% with a homogeneous particle size distribution [42]. The effect of TiO$_2$ addition on structure, bond type, and morphology was observed using X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). The use of different sources and synthesis methods is very likely to affect the structure, bonding, morphology, particle size, and effectiveness of the composite as an antibacterial agent [23,43].

2. Materials and Methods

2.1. Synthesis of HA-TiO$_2$ Composites by Hydrothermal Method

HA was synthesized by methods from our previous study [14,44]. HA was synthesized from CaO obtained from eggshells. Chicken eggshells were washed with water to remove contaminants and crushed using a planetary ball mill. After that, it was calcined at 1000 °C
for 5 h with an increase in temperature of 15 °C/min to produce CaO. The CaO obtained is then used as a precursor for the synthesis of HA by mixing it with diammmonium hydrogen phosphate ((NH₄)₂HPO₄, 99.0%, Merck, Kenilworth, NJ, USA) with a Ca:P mole ratio of 1.67. The pH of the solution with acetic acid (CH₃COOH, 99.5%, Merck, Kenilworth, NJ, USA) was adjusted to pH 9. Then, the mixture was put into an autoclave at 230 °C for 48 h. After that, the formed HA was dried to obtain HA crystals.

Composites were prepared by mixing HA and TiO₂ (Anatase, 99.0%, Merck, Kenilworth, NJ, USA) with various weight variations, namely HA:TiO₂ with ratios of 30:70 (H3T7), 40:60 (H4T6), 50:50 (H5T5), 60:40 (H6T4), and 70:30 (H7T3). The mixture was dispersed and stirred with 60 mL of deionized water using a magnetic stirrer, then put into a 100 mL autoclave, and then heated at 230 °C for 48 h to obtain a HA-TiO₂ composite [14,43]. The prepared synthesis process for HA-TiO₂ crystals is shown in Figure 1.

![Synthesis preparation for HA-TiO₂ composites by hydrothermal method.](image)

**Figure 1.** Synthesis preparation for HA-TiO₂ composites by hydrothermal method.

### 2.2. Composite Characterization

Composite structures (H3T7, H4T6, H5T5, H6T4 and H7T3) were determined with X-ray diffraction (XRD, PANalytical PW3040/X0 X’Pert PRO, Malvern, UK). Measurements were made in the range of 10–90 ° (2θ) with a step size of 0.01 ° and rate of 10 °/min using Cu Kα radiation (λ = 0.15406 nm) at room temperature. The XRD pattern was analyzed using HighScore Plus software (PANalytical 3.0.5) [45]. The crystallite size of the composite crystal is calculated by the Debye-Scherrer equation as in Equation (1) [46].

\[
D = \frac{(K\lambda)}{(B\cos\theta)}
\]  

where D is the crystal size (nm), K is the crystal form factor (0.9), λ is the wavelength of X-rays (0.15406 nm), B is the value of Full Width at Half Maximum (FWHM) (rad), and θ is angle of diffraction (rad). The crystallite size of HA was calculated from peaks at 2θ = 25.3°(101), while TiO₂ was calculated from peaks at 2θ = 25.3°(101). The crystallinity of synthesizing HA/TiO₂ was calculated from the XRD data using Equation (2) below [47]:

\[
\text{Crystallinity (\%)} = \left( \frac{I_{300} - V_{112/300}}{I_{300}} \right) \times 100\%
\]  

where \(I_{300}\) is the intensity of the diffraction peak at the (300), \(V_{112/300}\) is the intensity of the (112) and (300). In this study, the calculated SSA is crystalline SSA with the definition as surface area (SA) of crystals per mass of crystals [48]. SSA (m²/g) was calculated using Sauter’s formula (Equation (3)), where D is the crystallite size (m) and \(\rho\) is the density of crystals (g/m³).

\[
\text{SSA} = \frac{6}{(D \times \rho)}
\]
Ascertainment of the functional groups contained in HA-TiO₂ is by Fourier-transform infrared spectroscopy (FTIR, PerkinElmer Spectrum 100, Waltham, MA, USA) using the KBr disc technique, at a frequency interval of 400–4000 cm⁻¹ with a step size of 1 cm⁻¹. Each of the collected spectrums is an average of 16 FTIR scans. A few micrograms of the HA/TiO₂ composites were mixed with KBr, with a ratio KBr and composites 1:100, then pressed for structural analysis. To determine its morphology, composite samples were characterized using a scanning electron microscope (SEM, JEOL JSM-6360LA, Tokyo, Japan) with a voltage of 20 kV at an ×1000 magnification. Several milligrams of the composite were fixed in a sample holder and coated using gold. Then, the morphology and size were examined and analyzed using ImageJ 1.52a software [49]. A total of approximately 300 particles were analyzed for the area of each sample. The pixel area in the image is converted to the particle area. After that, the length/diameter of each particle was calculated. In addition, the mean and standard deviation values are also calculated.

2.3. Determination of Antibacterial Activity
2.3.1. Disc Diffusion Method
HA-TiO₂ composites are suspended to test their antibacterial properties on *E. coli* (Gram-negative bacteria) and *S. aureus* (Gram-positive bacteria). The method used is the disc diffusion method. All tools used for antibacterial testing are sterilized first by washing followed by heating using an autoclave at 121 °C for 120 min. After sterilization, the provision of test bacteria is then carried out, the test bacteria that have been inoculated are taken 1 isolate using sterile nichrome wire, then suspended into a test tube containing 5 mL of liquid media. Subsequently, the suspension of the test bacteria was incubated for 24 h at a temperature of 37 °C. Turbidity of the bacterial suspension was standardized to the equivalent of 1 McFarland. The media for nutrient agar (NA) is to be put in a petri dish and allowed to harden. The suspension of the test bacteria is taken and applied to the agar NA medium. Disc paper is placed regularly on the substrate so that it is then incubated for 24 h at a temperature of 37 °C. After that, observations are made and measured inhibition zones are formed using calipers. The inhibitory power is determined by reducing the diameter of the formed inhibition zone by the diameter of the disc paper (ø = 6 mm).

2.3.2. Optical Density Method
Antibacterial activity test using the optical density (OD) method measured the decrease in absorbance value at a wavelength of 600 nm. The composite sample, HA from eggshell, pure TiO₂, and positive control (4.5% amoxicillin), were prepared in distilled water. Then 3 mL of *S. aureus* and *E. coli* cultures (~10⁶ cfu/mL) were added to each. The mixed suspension was kept in an incubator with continuous shaking for different time periods. The results of incubation were measured for the absorption value (OD) at a wavelength of 600 nm using a UV-visible spectrophotometer [50]. The graph was plotted of OD at 600 nm versus time to predict the growth of bacteria after interacting with materials.

3. Results and Discussions
3.1. XRD Characterization
The XRD pattern of the HA-TiO₂ composite shows peaks corresponding to the Inorganic Crystal Structure Database (ICSD) 98-005-6311 for HA (hexagonal, P63/m) [51] and ICSD 98-015-4602 for TiO₂ anatase (tetragonal, I41/amd) [52], which is shown in Figure 2. The conformity of the sample peaks by reference confirms the presence of merging or composting [53]. The diffraction peaks at 2θ = 25.9°(002), 31.8°(211), 46.7°(222), and 49.5°(213) are typical peaks from pure HA, while the peak of anatase TiO₂ was evident at 2θ = 25.3°(101), 37.8°(004), and 48.0°(200). The highest peak of HA composite HA-TiO₂ resulted from the synthesis of H3T7, H4T6, H5T5, H6T4, dan H7T3 is the same as the highest peak of pure HA (31.8°). The peak intensity of HA increased with increasing HA concentration, as well as the peak of TiO₂ which increased with increasing TiO₂ content [17]. Based on the XRD pattern, all composites did not obtain other phase peaks. Thus, it can
be concluded that there is no impurity in the HA-TiO$_2$ composite. This indicates that no chemical reaction occurred that caused other products to form between the HA and TiO$_2$ phases.

Figure 2. XRD pattern of (a) TiO$_2$ ICSD 01-071-1166; (b) H3T7; (c) H4T6; (d) H5T5; (e) H6T4; (f) H7T3 and; (g) HA ICSD 00-009-0432.

The percentage of crystal structure was calculated by the Rietveld refinement method using HighScore Plus software (PANalytical 3.0.5). The goodness of fit (GoF) is calculated to confirm the accuracy of the Rietveld refinement. Figure S1 shows the Rietveld refinement of XRD pattern. The results show that the percentage of the phase formed in the XRD pattern corresponds to the amount of precursor added (Table 1). In addition, in this calculation, an accurate GoF value is obtained with a value close to 1 [54]. Then, the crystallinity of the sample was calculated based on the peaks in the XRD pattern. This crystallinity value is important because it determines its properties in further applications. It is known that HA-TiO$_2$ 50:50 composites have the highest crystallinity compared to other composites (Table 1). However, H3T7, H4T6, H5T5, and H6T4 composites had higher crystallinity than pure HA (56%) [44]. High crystallinity will cause the material to be more stable and have increased mechanical strength when applied [55,56].

Table 1. Percentage of phase composition, Rietveld refinement parameters, and crystallinity of the samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystal Phase (%) *</th>
<th>Rietveld Refinement Parameters</th>
<th>Crystallinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA</td>
<td>TiO$_2$</td>
<td>$R_{exp}$</td>
</tr>
<tr>
<td>H3T7</td>
<td>29.3 ± 0.0</td>
<td>70.7 ± 2.2</td>
<td>22.60</td>
</tr>
<tr>
<td>H4T6</td>
<td>40.1 ± 0.0</td>
<td>59.9 ± 2.6</td>
<td>22.59</td>
</tr>
<tr>
<td>H5T5</td>
<td>52.6 ± 0.0</td>
<td>47.4 ± 2.2</td>
<td>21.70</td>
</tr>
<tr>
<td>H6T4</td>
<td>61.2 ± 0.0</td>
<td>38.8 ± 2.8</td>
<td>23.33</td>
</tr>
<tr>
<td>H7T3</td>
<td>73.0 ± 0.0</td>
<td>27.0 ± 1.4</td>
<td>18.52</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation.
The crystallite size was calculated using the Debye-Scherrer equation. The crystallite size of HA was calculated from peaks at $2\theta = 31.8^\circ$ (211), while TiO$_2$ was calculated from peaks at $2\theta = 25.3^\circ$ (101). In Table 2, it can be seen that the crystallite size of the TiO$_2$ decreases with the increasing amount of TiO$_2$ in the composite. As for the crystallite size of HA, the highest crystallite size is in the H4T6 composite and tends to decrease in other composites. However, the difference is not significant. The calculation results show a relatively large measurement error with the difference between samples that are not too far apart. The smallest crystallite size for HA is in the H3T7 composite [57]. The crystallinity size will affect the specific surface area (SSA) properties. SSA is a very important material property for adsorption, heterogeneous catalysis, and reactions at the surface [58–60]. The H3T7 composite has the highest SSA value compared to other composites. This SSA value is directly proportional to the size of the crystallite obtained. The SSA values for HA and TiO$_2$ crystals are shown in Table 2.

Table 2. Crystallite size and specific surface area (SSA) calculated using Scherrer method from all peaks of HA and TiO$_2$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystallite Size (nm) *</th>
<th>SSA (m$^2$/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HA</td>
<td>TiO$_2$</td>
</tr>
<tr>
<td>H3T7</td>
<td>32.5 ± 2.9</td>
<td>13.5 ± 2.2</td>
</tr>
<tr>
<td>H4T6</td>
<td>42.8 ± 3.8</td>
<td>16.5 ± 3.4</td>
</tr>
<tr>
<td>H5T5</td>
<td>35.7 ± 3.8</td>
<td>13.6 ± 2.3</td>
</tr>
<tr>
<td>H6T4</td>
<td>39.2 ± 3.6</td>
<td>16.6 ± 3.3</td>
</tr>
<tr>
<td>H7T3</td>
<td>35.3 ± 7.1</td>
<td>17.5 ± 3.5</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation.

The HA crystal from the synthesized composite had a smaller lattice volume as the amount of TiO$_2$ was added (Table 3). This indicates that TiO$_2$ causes shrinkage of the lattice volume of HA. This phenomenon complies with another study we found demonstrating that the presence of TiO$_2$ can decrease the length of the lattice constant in the c direction [32]. The smallest HA lattice volume was obtained on the H3T7 composite with a volume of 528.211 Å$^3$. In addition, in the anatase TiO$_2$, the smallest lattice volume value is also shown in the H3T7 composite, which means that the small amount of TiO$_2$ gives the effect of decreasing the lattice volume. The volume of the TiO$_2$ anatase lattice in the H3T7 composite is 135.834 Å$^3$. The crystal structure of HA and anatase TiO$_2$ was shown in Figure 3.

Table 3. Crystal lattice parameters of the samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>HA *</th>
<th>Anatase TiO$_2$ *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$a = b$ (Å)</td>
<td>$c$ (Å)</td>
</tr>
<tr>
<td>H3T7</td>
<td>9.420(3)</td>
<td>6.873(3)</td>
</tr>
<tr>
<td>H4T6</td>
<td>9.422(3)</td>
<td>6.875(2)</td>
</tr>
<tr>
<td>H5T5</td>
<td>9.422(2)</td>
<td>6.875(2)</td>
</tr>
<tr>
<td>H6T4</td>
<td>9.425(2)</td>
<td>6.876(2)</td>
</tr>
<tr>
<td>H7T3</td>
<td>9.428(2)</td>
<td>6.879(2)</td>
</tr>
</tbody>
</table>

* Values in parentheses represent estimated standard deviations in the last quoted place.

3.2. FTIR Analysis

FTIR analysis was carried out to analyze the functional groups in the HA-TiO$_2$ composite. The FTIR spectrum of HA, TiO$_2$, and variations in the HA-TiO$_2$ are shown in Figure 4. The peak at wavenumber 3399–3571 cm$^{-1}$ for all HA-TiO$_2$ composites appears with a pointed shape indicating the presence of an OH group in HA [44]. Meanwhile, the peak at wavenumber 567–1091 cm$^{-1}$ is a response to the absorption for PO$_4^{3-}$ [61]. In the wavenumber range of 500–700 cm$^{-1}$, peak widening is seen, which is observed as the concentration of HA decreases [17]. Meanwhile, the peak at the wavenumber 1420–1458 cm$^{-1}$
is characteristic of the presence of a carbonate group (CO$_3^{2-}$). Carbonates identified on the FTIR spectrum can be atmospherically derived and adsorbed with HA [62,63]. The addition of TiO$_2$ to HA did not significantly show a shift in the FTIR spectrum. There is no bond between TiO$_2$ and HA, but that only indicates the presence of HA and TiO$_2$ that do not bind to each other, which is a characteristic of the formation of composites of both. HA-TiO$_2$ composite FTIR spectrum data for ratio variations can be seen in Table 4.

In the wave number range of 50–900 cm$^{-1}$, a point shape indicating the presence of a carbonate group (CO$_3^{2-}$) is characteristic of the formation of composites of both.

Carbonates identified on the FTIR spectrum can be atmospherically derived and adsorbed with HA [62,63]. The addition of TiO$_2$ to HA did not significantly show a shift in the FTIR spectrum. There is no bond between TiO$_2$ and HA, but that only indicates the presence of HA and TiO$_2$ that do not bind to each other, which is a characteristic of the formation of composites of both.

**Table 4.** Types of vibrations in HA, TiO$_2$, and HA/TiO$_2$ composites based on the peaks that appear in each wavenumber.

<table>
<thead>
<tr>
<th>Vibration Type</th>
<th>HA</th>
<th>TiO$_2$</th>
<th>H3T7</th>
<th>H4T6</th>
<th>H5T5</th>
<th>H6T4</th>
<th>H7T3</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti–O–Ti</td>
<td>-</td>
<td>800–450</td>
<td>800–450</td>
<td>800–450</td>
<td>800–450</td>
<td>800–450</td>
<td>800–450</td>
<td>[64]</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>1095–472</td>
<td>-</td>
<td>1091–744</td>
<td>1089 567</td>
<td>1089–600</td>
<td>1089–570</td>
<td>1089–567</td>
<td>[61]</td>
</tr>
<tr>
<td>OH free</td>
<td>-</td>
<td>1635</td>
<td>1635</td>
<td>1635</td>
<td>1634</td>
<td>1636</td>
<td>1639</td>
<td>[64]</td>
</tr>
</tbody>
</table>
3.3. SEM Analysis

Figure 5 shows the morphology of pure HA from eggshells and HA-TiO\(_2\) composite. The surface structure of HA samples from chicken eggshells has a surface shape in the form of agglomeration of fine sub-particles. After adding TiO\(_2\), relatively less agglomeration occurs. All composite morphologies have spherical and porous particle shapes. The entire composite sample looks inhomogeneous to each other. In addition, in each composite sample, there is a very large particle size that is agglomerated and fused with each other. The addition of TiO\(_2\) led to an increase in the density of the composite layer [53,57].

![Figure 5. SEM image of (a) HA from chicken eggshell; (b) H3T7; (c) H4T6; (d) H5T5; (e) H6T4; and (f) H7T3.](image)

From the particle size analysis using ImageJ 1.52a software (Figures S2 and S3) [65], all composites have different particle sizes between 90–1000 nm (Figure 6). Data regarding the value of the mean, mode, median, standard deviation, and polydispersity index (PI) are shown in Table 5. The amount of addition of TiO\(_2\) to the composite causes the average particle size to be smaller. Mode values of each composite variation H3T7, H4T6, H5T5, H6T4, and H7T3 were 90.8, 193.7, 194.1, 196.5, and 193.7 nm, respectively.
Figure 6. Particle size distribution from SEM image using ImageJ 1.52a software of (a) H3T7; (b) H4T6; (c) H5T5; (d) H6T4; and (e) H7T3.

Table 5. Statistical data calculation from particle size distribution using ImageJ from SEM image.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Measurement Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (nm)</td>
<td>Mode (nm)</td>
</tr>
<tr>
<td>H3T7</td>
<td>220.4</td>
<td>90.8</td>
</tr>
<tr>
<td>H4T6</td>
<td>344.2</td>
<td>193.7</td>
</tr>
<tr>
<td>H5T5</td>
<td>313.5</td>
<td>194.1</td>
</tr>
<tr>
<td>H6T4</td>
<td>292.2</td>
<td>196.5</td>
</tr>
<tr>
<td>H7T3</td>
<td>353.6</td>
<td>193.7</td>
</tr>
</tbody>
</table>

Furthermore, it is known that if the value of the polydispersity index (PI) is more than 1, it indicates that the sample has a wide nanoparticle distribution and non-uniform nanoparticle diameter size [66]. It is very difficult to make particles of uniform size (monodispersion). The PI values for the H3T7, H4T6, H5T5, H6T4, and H7T3 composites were 1.42, 1.42, 1.32, 1.33, and 1.31, respectively. The H3T7 composite has the smallest average particle size. It is
understood that the amount of TiO$_2$ affects the sample size, whereas the addition of TiO$_2$ can cause a decrease in particle size. Size reduction can increase the redox rate for electrons and holes during surface photocatalytic processes. It also decreases photoelectron and hole recombination thereby increasing reactivity [66].

### 3.4. Antibacterial Activity

The results obtained from this antibacterial test are inhibitory diameter which can be seen in Figure 7. The inhibition zone showed bactericidal activity on the Gram-positive bacteria (S. aureus) and Gram-negative bacteria (E. coli). The highest inhibition zone is found in the H3T7 composite, where Gram-positive bacteria are more extensive than Gram-negative bacteria. These results correspond to previous studies that reported that the antibacterial activity of the HA-TiO$_2$ composite is more pronounced against Gram-positive bacteria due to the plasmolysis of the cell wall or the separation of the cytoplasm from the cell wall [40]. Some studies have reported that Gram-negative bacteria are usually more resistant. This resistance is associated with the cell walls of Gram-negative bacteria that are more complex than those of Gram-Positive bacteria. However, under certain conditions, Gram-negative bacteria can be more resistant to chemical agents than to Gram-positive bacterial cells [67,68]. Another reason is that the external membrane of Gram-negative bacteria consists mainly of a strong layer of lipopolysaccharides, which are considered a barrier. Thus, Gram-positive bacteria have a high sensitivity due to differences in the composition of their cell walls compared to Gram-negative bacteria [69,70]. The antibacterial test image can be seen in Figure S4.

![Inhibition zone test of HA-TiO$_2$ composite against Escherichia coli and Staphylococcus aureus bacteria.](image)

**Figure 7.** Inhibition zone test of HA-TiO$_2$ composite against *Escherichia coli* and *Staphylococcus aureus* bacteria.

Antibacterial testing on TiO$_2$ does not produce an inhibitory zone. In contrast to TiO$_2$, HA in the test produces an inhibitory zone, this is because HA can adsorb bacteria, in other words, HA deactivates bacteria by an adsorption mechanism. Adhered bacteria are believed to be killed due to the disruption of their cell membrane by the composites, which reach across the microbial membrane [71]. However, the combination of compounds, namely the HA-TiO$_2$ composite, makes the inhibition zone vary. Since this antibacterial activity is a surface reaction [72,73], this activity is determined by surface area and particle size. Samples with higher surface area and small particle sizes showed greater antibacterial activity.

After that, the composite antibacterial activity test was carried out using the optical density method. The positive control used was amoxicillin and the negative control was S. aureus and E. coli without the addition of composites. Amoxicillin was chosen as a positive control in the antibacterial activity test because amoxicillin is an antibiotic that is widely
used in the treatment of various infectious diseases caused by pathogenic bacteria [74]. In this study, the difference between the OD method and the disc diffusion method is that in the OD method, light is used to initiate the photocatalytic process. Figure 8 shows the photocatalytic antibacterial activity based on the percentage of inhibition of *E. coli* and *S. aureus* bacteria. All composites applied to each bacterium were recorded in time intervals. Based on the test results, the antibacterial activity of the composite was higher than pure HA, pure TiO$_2$, and the positive control of amoxicillin. The greatest inhibition was obtained from the H3T7 composite for *E. coli* bacteria. In addition, the H3T7 composite also has the smallest particle size and the largest specific surface area, thereby increasing the active site and surface reactivity. H3T7 composites have high antibacterial activity compared to other composites, this is supported by XRD data showing a smaller crystalline size and high surface area, and by SEM data which shows smaller particle sizes [75,76]. A large specific surface area will lead to an increase in the active site and surface reactivity [72]. However, for *S. aureus* bacteria, the best antibacterial activity was shown by the H7T3 composite followed by H3T7. This is in accordance with the antibacterial data using the disk diffusion method that the H3T7 and H7T3 composites provide the largest zone of inhibition. The growth of bacteria decreased drastically over the period. This study demonstrates that both materials’ synergistic effect in a short time is well proven.

**Figure 8.** Optical density measurements of bacteria at a wavelength of 600 nm (OD 600) for (a) *Escherichia coli*; and (b) *Staphylococcus aureus*.

### 4. Conclusions

HA-TiO$_2$ composites from eggshells have been successfully synthesized using the hydrothermal method. It can be concluded that the composition of the HA-TiO$_2$ composite from eggshell affects the crystallinity, crystal size, specific surface area, and particle distribution. The H3T7 composite had the smallest crystallite size and the highest specific area, namely 32.5 ± 2.9 nm and 58.4 m$^2$/g for HA, and 13.5 ± 2.2 nm and 113.7 m$^2$/g for TiO$_2$, respectively. The FTIR spectrum shows that there is no chemical bond formed between TiO$_2$ and HA which indicates the formation of a composite. The morphology of the HA-TiO$_2$ composite has an irregular particle shape, and agglomerates, and is not homogeneous for all compositions. The size distribution shows that the H3T7 composite has the smallest particle size. This indicates that the addition of TiO$_2$ to HA significantly reduces the particle size.

Antibacterial activity using the disk diffusion method showed that the H3T7 composite had the highest antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* bacteria compared to other composites. In addition, the optical density (OD) method also showed that all composites had better antibacterial activity than pure HA and pure TiO$_2$. These results indicate that the synergistic combination of HA from eggshell with TiO$_2$ has favorable properties for antibacterial activity.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cryst12111599/s1, Figure S1: Rietveld refinement of XRD pattern of (a) H3T7; (b) H4T6; (c) H5T5; (d) H6T4; and (e) H7T3; Figure S2: SEM image with ×10,000 magnification of (a) H3T7; (b) H4T6; (c) H5T5; (d) H6T4; and (e) H7T3; Figure S3: SEM Image after processing in ImageJ 1.52a software for particle distribution analysis of (a) H3T7; (b) H4T6; (c) H5T5; (d) H6T4; and (e) H7T3; Figure S4: Antibacterial test results against Escherichia coli (1) and Staphylococcus aureus (2) bacteria using disc diffusion method of (a) HA; (b) TiO2; (c) H3T7; (d) H4T6; (e) H5T5; (f) H6T4; and (g) H7T3.

Author Contributions: Conceptualization, A.R.N. and D.R.E.; methodology, E.N.A.; software, E.N.A. and M.D.P.; validation, A.R.N. and D.R.E.; formal analysis, A.R.N.; D.D. and M.D.P.; investigation, E.N.A.; D.D. and M.D.P.; resources, E.N.A.; data curation, E.N.A.; writing—original draft preparation, E.N.A.; writing—review and editing, E.N.A. and M.D.P.; visualization, E.N.A. and M.D.P.; supervision, A.R.N. and D.R.E.; project administration, A.R.N. and S.; funding acquisition, A.R.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Riset Data Pustaka dan Daring (RDPD) 2021, Universitas Padjadjaran, grant number 1959/UN6.3.1/PT.00/2021.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank to Kiki Maesaroh for preparing the materials. We also thank to Annisa Luthfiah and Lintang Kumoro Sakti for discussing the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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