Evaluation of antibacterial activity of nano-hydroxyapatite (HAp) from freshwater mussel (Pilsbryoconcha sp.) shell against Escherichia coli

Santhy Wisuda Sidauruk1*, Dian Iriani1, N Ira Sari1, Rizky Ramadhan Rusdi1, Muhammad Irfan Rusadi1

1Dept. of Fish Product Technology, Faculty of Fisheries and Marine Science, Universitas Riau, Indonesia

Abstract. Freshwater mussel shells have a high calcium content as a precursor in the synthesis of hydroxyapatite (HAp). The main components of HAp are calcium and phosphate, which can prevent dental plaque bacteria, such as Escherichia coli. This research aimed to evaluate the antibacterial activity of nano-hydroxyapatite (nano-HAp) from Pilsbryoconcha sp. against E. coli. The procedure for preparing nano-HAp consisted of producing CaO flour through a calcination process and producing nano-HAp using the bottom up method. The test parameters consisted of the yield and evaluation of antibacterial activity at different concentrations of nano-HAp, namely 50 mg/mL, 25 mg/mL, and 12.5 mg/mL. The results showed that the yield of nano-HAp was 65.13±0.83%. Based on antibacterial activity, nano-HAp had an inhibition zone against the bacteria that causes dental plaque E. coli. The diameters of the inhibition zones of nano-HAp against E. coli bacteria at concentration of 12.5 mg/mL, 25 mg/mL, and 50 mg/mL were relatively weak, those were 1.23±0.31 mm, 2.51±0.38 mm, and 3.77±0.28 mm, respectively. The antibacterial activity of nano-HAp can be increased through modification with metal doping or natural materials with antioxidant or antibacterial activity.

1 Introduction

Freshwater mussel (Pilsbryoconcha exilis) is a shellfish that has not been utilized optimally. The local community only uses freshwater mussel meat as food because it is rich in essential amino acids, fatty acids, a low cholesterol content, and minerals [1]. In fact, processing freshwater mussel produced large quantities of solid waste in the form of shells. The solid waste produced from mussels was 51.90% of the total weight of freshwater mussel [2]. More freshwater mussel production will result in more freshwater mussel shell waste, which could negatively affect humans and the environment. The potential for solid waste resulted from using freshwater mussels is so significant that it is necessary to make a breakthrough to maximize the utilization of freshwater mussel shells.

Freshwater mussel shells were rich in minerals and exceptionally high in calcium content.
The high calcium content in freshwater mussel shells could be utilized in making freshwater mussel shell flour, which is applied to mineral-rich cookies [3][4]. Moreover, the high calcium in freshwater mussel shells has been utilized by converting it into Calcium Oxide (CaO) flour, which functioned as a calcium precursor in the synthesis of hydroxyapatite using the calcination method with a calcium content of 76.27% [2].

Hydroxyapatite (HAp) consists of the mineral compound apatite (Ca10(PO4)6(OH)6) which can be synthesized using high-calcium material which can potentially form human bones and teeth because the main components of HAp are calcium and phosphate [5]. Calcium and phosphate compounds also play a role in preventing dental caries [6]. Dental caries occurs due to repeated consumption of simply fermentable carbohydrates, mainly sucrose, causing the overgrowth of cariogenic bacteria [7]. Dental caries occurs due to the formation of plaque biofilm on the teeth [8]. Dental plaque was a microbial community that colonized the tooth surface in a structured and organized biofilm [9]. One of the bacteria that caused dental plaque was *Escherichia coli* [10][11].

The synthesis of nano-sized hydroxyapatite had very promising potential because the small particle size facilitated penetration into bacterial cells and provided maximum contact with the environment [12]. Furthermore, using nano-sized particles had several advantages, namely, increased absorption, reduced dose required, and increased stability [13]. As of this research, it was necessary to conduct research on the synthesis of nano-HAp from freshwater mussel shells and its characteristics on the antibacterial effectiveness of *Escherichia coli* as dental plaque bacteria that triggered dental caries. The research aimed to evaluate the antibacterial activity of nano-hydroxyapatite (HAp) from *Pilsbryoconcha* sp. shell against *Escherichia coli*.

## 2 Materials and methods of research

### 2.1 Materials and Equipment

The materials used in this research as a source of calcium in the synthesis of nano-HAp were *Pilsbryoconcha* sp. shells (2 kg) obtained from Sungai Paku, Riau Province and diammonium hydrogen phosphate NH4H2PO4 (Merck, Germany), PBS (Phosphate Buffered Saline) solution, *Escherichia coli* bacterial isolated from the Pekanbaru Regional Health Laboratory, Mueller Hinton Agar media (HIMEDIA, USA), Nutrient Agar media (HIMEDIA, USA), the antibiotic ciprofloxacin, and other chemicals used for analysis.

The equipment used in the preparation of *Pilsbryoconcha* sp. shells, the production of *Pilsbryoconcha* sp. shell flour, the production of CaO flour as a source of calcium, and the synthesis of nano-HAp include muffle furnaces, digital scales, porcelain cups, measuring cups, pH meters, mortars, magnetic stirrer, hot plate, thermometer, petri dish (CMSI Normax), loop needle, incubator (Precision scientific), autoclave (Hiclaveb HVE-50), erlenmeyer (Iwaki pyrex), test tube (Iwaki), Bunsen, and caliper.

### 2.2 Production of CaO Flour

*Pilsbryoconcha* sp. shells are washed using distilled water to release adhering dirt, dried in the sun, floured, and sifted. *Pilsbryoconcha* sp. shell flour is calcined to get CaO flour. Freshwater mussel shells were calcinated at 1000°C for 6 hours [14].

### 2.3 Production of Nano-Hydroxyapatite

Synthesis of nano-HAp was conducted using the bottom-up method [15]. Started by reacting 1 M CaO solution with 0.6 M NH4H2PO4 solution, stirred for 1 hour at 90°C, then controlled the pH to 10 using 1 M NaOH, then a precipitation process for 18 hours. The precipitate was neutralized using distilled water until the pH was neutral, then centrifuged at 4500 rpm for 15 minutes. Next, the precipitate was dried in an electric oven for 72 hours at
65°C and calcined at 1000°C with a temperature increase rate of 10°C/minute for 2 hours in a furnace and cooled in a desiccator for 1 hour [16]. Subsequently, nano-HAp of freshwater mussel shells was obtained, which was evaluated for the antibacterial activity of *E. coli*.

2.4 Yield

The yield of nano-HAp was calculated by comparing the weight of nano-HAp obtained with the weight of CaO flour used.

2.5 Antibacterial Activity Evaluation

This study evaluated antibacterial activity using the Kirby-Bauer disc diffusion method to assess the inhibition zone of bacterial growth. Initially, Mueller Hinton Agar (MHA) media 20 mL is decanted into a petri dish and cooled in the refrigerator for 2x24 hours until it solidifies [17]. Bacterial colonies were inoculated into 5 mL of Nutrient Agar (NA) media at 1.5x10^8 CFU/mL for 2x24 hours. The bacterial inoculum was dispersed using distilled water into the bacterial suspension to obtain a suspension turbidity that met the McFarlan standard of 0.5 [18]. The bacterial suspension was wiped using a sterile cotton bud, and the culture method was tilted by streaking in a zig-zag manner over the entire MHA medium. Sterile paper discs at 6 mm were placed in Petri dishes. The test material was nano-HAp with 12.5 mg/mL, 25 mg/mL, and 50 mg/mL, which were dropped onto a paper disc. PBS solution was used as a negative control, and ciprofloxacin was used as a positive control. Petri dishes were incubated at 37°C for 24 hours in an incubator. A caliper was used to measure the bacterial inhibition zone in millimeters (mm) [19]. The condition of potential bacterial inhibition is determined by the appearance of a clear zone (inhibition zone) around the paper disc. The clear zone formed is measured vertically and horizontally.

2.6 Statistical Analysis

The yield values were determined descriptively using Microsoft Excel, presenting the average ± standard deviation. Data on the diameter of the inhibition zone for *E. coli* bacteria were analyzed using SPSS IBM Software with ANOVA statistical analysis (Analysis of Variance) at a confidence level of 95% and Duncan's test. The experimental design resulting from the analysis of the antibacterial activity of *E. coli* was a completely randomized design with three nano-HAp concentration treatments consisting of 50 mg/mL, 25 mg/mL, and 12.5 mg/mL.

3 Results

3.1 Characteristics of Nano-Hydroxyapatite

The freshwater mussel has been identified as *Pilsbryoconcha exilis* included in the Family of Unionidae based on morphological and physiological characteristics presented in Figure 1.

![Fig.1. A species of mussel gastropod (*Pilsbryoconcha exilis*)](image)

The freshwater mussel *P. exilis* habitat is freshwater waters spread across Indochina, Sumatra, Java, Borneo, and Southeast Asia. The shell is yellow to brownish and elongated, reaching 10 cm in size. Besides, the spinal segments of *P. exilis* often have distinctive angles.
Usually, this species is found confined to small rivers and rice fields with muddy/sandy sediments [20].

Dried freshwater mussel shells have been separated from the meat, floured, calcined at 1000°C, and nano-HAp synthesized using the bottom-up method are presented in Figure 2.

![Figure 2](image_url)

**Fig 2.** (a) Freshwater mussel shell flour, (b) CaO flour, (c) nano-HAp of freshwater mussel shells

Initially, freshwater mussel shell flour was brownish-white. After calcination at 1000°C produced CaO flour, which was brownish. It is caused by high temperatures, which will cause the loss of organic compounds such as fat, protein, and carbon, which cause a material's color to become less white [21]. The calcination process is closely related to color changes. The brownish profile of CaO flour indicated that the hydrocarbon compounds from organic and carbonate residues have yet to be wholly decomposed.

Lastly, Nano-HAp produced using the bottom-up method was bluish-white because the bottom-up method is carried out with a solution maintained at pH 10 using NaOH to prevent the formation of calcium dehydrate and calcium monophosphate, which are readily soluble in water [22]. pH control was necessary to produce pure hydroxyapatite. Generally, the pH value was between 10 and 10.5 [23]. Conditions of pH 10 or alkaline pH during the synthesis process will cause many OH− and PO₄³⁻ groups to form, indicating pure hydroxyapatite [6]. Nano-HAp in alkaline conditions caused a slightly bluish color. Meanwhile, the white color indicated that the material contained calcium [24].

### 3.2 Yield

Yield was one of the analyses carried out to determine the percentage of the amount of hydroxyapatite produced from the CaO flour of *P. exilis* shells. The yield value of nano-HAp is presented in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nano-Hydroxyapatite</th>
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<tbody>
<tr>
<td>Freshwater Mussel Shell (Bottom up method)</td>
<td>65.13±0.83</td>
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<tr>
<td>Tuna Bones (Top down method) [25]</td>
<td>57.73</td>
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</table>

The yield of nano-HAp from freshwater mussel shells using the bottom-up method was 65.13% higher than nano-HAp from tuna bones using the top-down method due to the difference in making nanoparticles, namely the bottom-up method utilizing an approach of assembling atoms or molecules, then combining them through a chemical reaction, for example using the precipitation method. Meanwhile, the top-down method reduced large materials, such as a milling tool [26]. The sintering temperature will also affect the yield value obtained.
The high sintering temperature will remove all organic components in nano-HAp into a purer mineral form [21]. Apart from that, the yield decreased due to the sintering process, allegedly due to the loss of water content and organic material in hydroxyapatite [27].

Decreased yield value based on thermogravimetric analysis consisted of three temperature inflection points, namely at a temperature of 30-75°C was the first inflection point that caused the removal of water content. The second inflection point was at a temperature of 280-340°C is the point of removing organic composition. The third inflection point at 640-780°C was the decomposition point of the CaCO₃ structure into CaO [28].

3.3 Antibacterial Activity of E. coli on Nano-Hydroxyapatite

The antibacterial inhibition of *E. coli* (gram-negative bacteria) on nano-HAp is presented in Table 2. *E. coli* bacteria is gram-negative, non-spore-forming, motile, flagellum-shaped, rod-shaped, able to survive in simple media, producing gas and acid from glucose, fermenting lactose, and as dental plaque.

<table>
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<tr>
<th>Nano-HAp of freshwater mussel shell</th>
<th>Inhibition</th>
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<tr>
<td>50 mg/mL</td>
<td>+</td>
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<tr>
<td>25 mg/mL</td>
<td>+</td>
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<tr>
<td>12.5 mg/mL</td>
<td>+</td>
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</table>

Table 2 showed that nano-HAp of freshwater mussel shells inhibited the growth of *E. coli* bacteria at 50 mg/mL, 25 mg/mL, and 12.5 mg/mL. The formation of a clear zone indicated this inhibition. Antibacterial activity was determined from the diameter of the inhibition zone formed (mm) in Figure 3.

![Figure 3](image_url)

Information: Different superscript letters indicated significant differences (p<0.05). Each bar reflected the mean ± SD (standard deviation) of three independent test.

**Fig.3.** Antibacterial activity of nano-HAp against *E. coli*

The diameter of the inhibition zone for *E. coli* bacteria increased as the concentration of nano-HAp in freshwater mussel shells increased. The largest diameter of the inhibition zone for *E. coli* bacteria was found in 50 mg/mL nano-HAp of 3.77 ± 0.28 mm. Nano-HAp at 12.5 mg/mL, 25 mg/mL, and 50 mg/mL was able to inhibit the growth of *E. coli* bacteria as indicated by the formation of clear zones of 1.23 ± 0.31 mm, 2.51 ± 0.38 mm, and 3.77 ± 0.28 mm.
The antibacterial activity of nano-HAp from P. exilis shells was higher than the antibacterial activity of hydroxyapatite from duck eggshell waste without antibacterial activity against the bacteria Streptococcus mutans, Nocardia asteroides, Nocardia erythropolis, and Lactobacillus acidophilus [6]. Commercial-HAp, filtering-HAp, and filtering-HAp using H2O2 had no antibacterial activity against Staphylococcus aureus gram-positive bacteria and E. coli gram-negative bacteria [18], pure HAp also had no antibacterial activity against S. epidermidis, S. aureus, Pseudomonas aeruginosa, and E. coli bacteria [29]. The bacterial inhibition zone formed is less than 5 mm and has a weak level of inhibition of bacterial growth. The bacterial inhibition zone ranges from 5-10 mm to the medium category, 10-20 mm to the strong category, and more than 20 mm to the powerful category [30].

Nano-HAp had better antibacterial activity than HAp without antibacterial activity because nano-sized hydroxyapatite facilitated penetration into bacterial cells and provided maximum contact with the environment. However, bacterial growth inhibition level in nano-HAp was in the weak category. It meant that nano-hydroxyapatite's chemical interaction ability was not strong enough to damage the bacterial cell membrane layer [31]. So, to increase the antibacterial activity of nano-HAp, it is crucial to modify it with metal doping [31] or natural materials containing antioxidant activity or antibacterial activity to improve the chemical interaction ability of nano-HAp.

Chemically, HAp particles are known as Ca structures covered in phosphate; therefore, the chemical interaction is weak towards the outside of the crystal because Ca and P have formed a strong bond, and the potential difference is equal, so the chance of chemical interaction to the outside is minimal, which results in the weak antibacterial activity of pure HAp [32].

4 Conclusions

Based on its antibacterial activity, nano-HAp had an inhibitory zone against E. coli (gram-negative bacteria) as an act dental plaque. The diameter of the nano-HAp inhibition zone against E. coli bacteria was relatively weak, namely 1.23 ± 0.31 mm at 12.5 mg/mL nano-HAp, 2.51 ± 0.38 mm at 25% nano-HAp, and 3.77 ± 0.28 mm at 50% nano-HAp. The antibacterial activity of nano-HAp is enhanced through modification with metal doping or natural materials containing antioxidant or antibacterial activity.

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References