Effect of hydroxyapatite from waste of tilapia bone
(oreochromis niloticus) on the surface hardness of enamel

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Abstract. Demineralization is the process of removing calcium and phosphate ions from hydroxyapatite crystals in hard tissues of the teeth. The lost calcium and phosphate ions can be replaced by application of a remineralization agent ie hydroxyapatite. Hydroxyapatite can be synthesized from natural ingredients such as fish bones. In this study was to determine the effect of hydroxyapatite from bone waste of tilapia fish (Oreochromis niloticus) on hardness of enamel surface. Thirty-two premolar teeth were mounted on self cured acrylic resin and were divided into 4 groups and demineralized. Enamel hardness (pre-test) was measured by Vickers Hardness Tester and followed by a process of remineralization using a hydroxyapatite paste (HA) that synthesized from the tilapia bone by precipitation method. The paste was applied to group A (5% HA), B (10% HA), C (15% HA) then was immersed in artificial saliva while group D (control) was only immersed in artificial saliva. The surface hardness of enamel was measured again (posttest) after 7 days of treatment. The surface hardness of enamel for group A (424,19±14,50); B (456,58±9,82); C (482,64±9,90); D (418,44±8,23). Statistical tests showed that there were significant differences between the treatment group and the control group except group A. Application of hydroxyapatite paste from bone waste of the tilapia (Oreochromis niloticus) could increase the surface hardness of the enamel.

1. Introduction
Caries is a process of destruction of dental tissue by bacteria, either enamel or cementum which is demineralized by bacterial acid. Demineralization is the process of the disappearance of hydroxyapatite crystals which are composed of calcium and phosphate ions because of the difference in pH between the outer and inner surfaces of the enamel, which if it occurs continuously, forms a cavity on the surface of the enamel and reduce surface hardness of enamel [1]. Calcium and phosphate ions which are lost due to demineralization are reversible so that when the pH of the oral cavity is neutral and there are enough calcium and phosphate ions, the lost ions can be returned to the tooth structure [2]. Calcium and phosphate can be obtained by applying hydroxyapatite on enamel surfaces. Hydroxyapatite is composed of calcium and phosphate, therefore it can act as a reservoir. Some researchers have explained about the use of hydroxyapatite in increasing tooth remineralization. Sandiasa et al. reported that the addition of hydroxyapatite in toothpaste can improve the remineralization process of dentin [3]. Other studies also showed similar results when the teeth that
had enamel lesions immersed for 3-15 days with a nano hydroxyapatite solution at a 10% concentration showed an increase in microhardness [4]. Hydroxyapatite can be obtained from natural materials such as egg shells, beef bones, and fish bones. Mustafa et al. reported that hydroxyapatite was synthesized from tilapia bones and scales formed when heated at 900°C-1000°C [5]. Hydroxyapatite was synthesized from fish bones is biocompatible. This is evidenced by the research of Pal et al. who perform cytotoxicity testing on hydroxyapatite from fish bones. The results showed no cell death during the study [6]. The volume of tilapia fish on Indonesia production reached 328,473 tons (in 2011) and increased to 338,659 tons (in 2012) [7]. The high amount of tilapia processed will cause bone waste produced also to increase. Fish bones are only used as a mixture of animal feed or are usually thrown away. Therefore, it is necessary to make efforts to utilize fish bone waste into something useful. This study aims to determine the effect of hydroxyapatite from tilapia bone waste on surface hardness of enamel.

2. Methods

2.1. Sample preparation

The subjects of this study were 32 extracted premolar teeth, free from caries and fractures. The crown of the tooth is separated from the root of the tooth at the cemento enamel junction (CEJ) by cutting horizontally. The separated crown is then cut vertically in the mesiodistal direction so that it separates the crown into two parts, the buccal and lingual parts (figure 1) [8,9]. The buccal section was implanted in self cure acrylic resin using a tubular mold with a diameter of 1.5 cm and a height of 2 cm. The enamel surface is flattened using 400,1000,1500 size sandpaper in sequence [10].

![Figure 1](image)

Figure 1. Sectioning of the tooth vertically in mesiodistal direction.

All specimens were the immersed in demineralization solution for 48 hours at 37°C. The demineralization solution was prepared by mixing 2.2 mM CaCl₂, 2.2 mM NaH₂PO₄·7H₂O, 0.05M lactic acid, pH 4.5 was adjusted by adding 50% NaOH [11]. After the demineralization process was completed, all specimens were rinsed in aquades and dried. Thirty-two specimens formed were randomly divided into four group (A, B, C, D) of 8 teeth each. Then, the surface of samples was dried and the initial microhardness (pre-test) was measured by means of a Vickers indentor with 10 kg of force for 5 seconds [11]. Three indentations per test were performed on each specimen during each experimental stage and the mean was taken.

2.2. Fish bone preparation

Tilapia is boiled in 2 hours duration in order to remove the adherent fish meat [5]. Then, the bone were dried at least two day to make sure the removal of water and organic portion from boiled process are left behind. Dried tilapia bone was mashed using ball milling. Samples that have been finely calcined using furnace at 1000°C for 5 hours [5]. The calcination process is carried out to eliminate organic components and other metals than Ca and decompose calcium carbonate (CaCO₃) into calcium oxide (CaO) which will be used as Ca precursors to produce the hydroxyapatite powder [5].

2.3. Synthesis of hydroxyapatite

Hydroxyapatite synthesis is carried out using precipitation method by mixing CaO powder derived from fish bones with HNO₃ solution to produce calcium nitrate (Ca(NO₃)₂) solution. The calcium
nitrate solution that has been formed is mixed with phosphoric acid (H₃PO₄) solution while heating at 40°C with a speed of 300 rpm [12,13]. The pH of the previous solution was measured first, after that it was set at pH 10 by adding ammonium hydroxide (NH₄OH). The aging process is carried out for 24 hours to produce precipitate. The precipitate is then filtered and washed with distilled water to remove byproducts namely ammonium nitrate (NH₄NO₃) [13,14]. Precipitates are dried using an oven at 110°C for 5 hours. Dry precipitates are re-sintered using a furnace at 900°C for 5 hours [5].

2.4. Characterization of Hydroxyapatite Particles
The characterizations of hydroxyapatite powder after calcination process were analyzed by X-ray Diffraction (XRD) to identify the mineralogy of sample powder and use to confirm the purity and stability phase of sample powder.

2.5. Preparation of hydroxyapatite paste
Na-CMC was developed using distilled water and stirred until homogeneous. Hydroxyapatite powder which has been tested using XRD is mixed with glycerin and water. Glycerin mixture is added slowly into Na-CMC which has been developed while stirring until homogeneous [15].

2.6. Sample treatment
Teeth were dried with air spray. Hydroxyapatite paste with different concentrations applied to each teeth sample in each group, group A (5% hydroxyapatite paste), group B (10% hydroxyapatite paste), group C (15% hydroxyapatite paste) and group D (control group, only immersed in artificial saliva). Teeth that has been applied with paste hydroxyapatite then immersed with artificial saliva for 24 hours. This procedure is carried out every day for 7 days and by replacement of artificial saliva. After finishing the sample treatment for 7 days, an enamel surface hardness test was carried out using the Vickers Hardness Tester (Posttest).

3. Results

3.1. X-ray diffraction analysis
Characterization of hydroxyapatite powder using XRD after calcined at 900°C for 5 hours showed the highest peaks in the diffractogram with (2θ) 31.32; 32.00; 32.32 for hydroxyapatite (Figure 2), 27.78; 32.21 for calcium (Figure 3) and 20.42; 30.84; 34.73 for tricalcium phosphate (Figure 4).

Characterization using XRD was carried out qualitative analysis by comparing the peaks formed on hydroxyapatite diffractogram resulting from synthesis with standard hydroxyapatite diffractogram from JCPDS data (Joint Committee on Powder Diffraction Standards) No. 09-432 with a value of 2θ equal to 25,879; 31,809; 32,196; 32,902, and 34,048 [14]. After being compared, it can be concluded that the hydroxyapatite diffractogram synthesized has a value of 2θ which is almost close to the standard diffractogram value of about 31.32; 32.00 and 32.32. Therefore it can be concluded that the powder is hydroxyapatite. In addition to the formation of hydroxyapatite compounds also found the presence of other compounds such as tricalcium phosphate (TCP) and calcium (Ca) with each compound percentage of 54.15% for hydroxyapatite; 27.27% for tricalcium phosphate; and 18.58% for calcium. The percentage of each compound was obtained from the results of quantitative analysis carried out on the sample.

The data obtained from this study are normally distributed and homogeneous. Paired t-test test results in Table 1. showed that there were significant differences in enamel hardness values (p<0.05) before and after the application of remineralization material in the form of hydroxyapatite paste in groups A, B, C, and artificial saliva in group D (control). One way Anova test showed that there were significant differences in enamel surface hardness. Bonferroni test in Table 2. It shows that there is a significant difference in enamel hardness values (p<0.05) between groups which added various hydroxyapatite concentrations, such as between group A (5% HA) and group B (10% HA), group C (15% HA), between group B (10% HA) with group C (15% HA), group D (control), between group C (15% HA) with group D (control). Group A with the addition of 5% hydroxyapatite did not show a significant difference in the value of enamel hardness with group D (control).
4. Discussions
Hydroxyapatite is the main biomineral component found in human hard tissues such as teeth and bones. Hydroxyapatite used in this study was sourced from tilapia bones synthesized using precipitation method. The hydroxyapatite powder produced was tested using XRD to determine the composition of the compounds contained in the powder. This research was conducted by mixing tilapia bone powder with HNO$_3$, H$_3$PO$_4$ and NH$_4$OH solutions then calcined at 900°C. According to Monmaturapoj, calcined at temperatures above 800°C can result in the formation of other phases such as tricalcium phosphate (TCP), while the calcium ions that are read at XRD examination is thought to originate from impurities in the starting material [16,17].

![Figure 2. Diffractogram results of XRD analysis for hydroxyapatite.](image1)

![Figure 3. Diffractogram results of XRD analysis for calcium.](image2)
Figure 4. Diffractogram results of XRD analysis for tricalcium phosphate.

Figure 5. Average and standard deviation (SD) of early enamel hardness (pre-test) and after remineralization (post-test).

Table 1. Enamel hardness before and after application remineralization material.

| Group | P Value |
|-------|---------|
| Group A | .000 |
| Group B | .000 |
| Group C | .000 |
| Group D | .000 |

Based on the results of the paired t-test (Table 2) comparing the hardness of enamel before and after hydroxyapatite paste application showed that there were significant differences in the value of enamel hardness all groups. Increasing the enamel hardness in groups A, B, and C was caused by the application of remineralization material such as hydroxyapatite paste, enamel hardness on group D that immersed in artificial saliva (control) also increased. Artificial saliva used in this study contains minerals needed for the remineralization process, such as calcium and phosphate.
Table 2. Differences in enamel surface hardness between groups.

| Group | A (5% HA) | B (10% HA) | C (15% HA) | D (Control) |
|-------|-----------|------------|------------|-------------|
| A (5% HA) | 1,000 | 0,000 | 1,000 | 1,000 |
| B (10% HA) | 0,000 | 1,000 | 0,000 | 0,000 |
| C (15% HA) | 0,000 | 0,000 | 1,000 | 0,000 |
| D (Control) | 0,000 | 0,000 | 0,000 | 0,000 |

The increase in enamel hardness that occurred in this control group was in accordance with the results of the study by Ionta et al. that artificial saliva can remineralize the demineralized enamels [18]. Haghgou et al. also showed similar results, the value of enamel hardness increased after immersion in artificial saliva for 10 minutes. Artificial saliva is able to maintain saturated oral cavity environment of calcium and phosphate ions make provide protection to hydroxyapatite crystals and the ions could not released from enamel [19]. The increasing enamel hardness that occurs in the treatment group is affected by the components present in hydroxyapatite paste such as calcium and phosphate. When the enamel demineralizes, calcium and phosphate ions come out from the hydroxyapatite crystals and make enamel hardness decrease. The calcium and phosphate content in the hydroxyapatite paste will diffuse into the enamel structure to form a new apatite layer which increases the hardness of the enamel surface [20]. This is also supported by the research of Haghgoo et al. that when teeth with enamel lesions were given toothpaste containing nano hydroxyapatite for 5 days showed an increase in microhardness enamel [4]. Increasing microhardness occurs due to the penetration of hydroxyapatite into the porosity so that it closes the pores on the enamel surface. Hydroxyapatite also acts as a scaffold so it can help absorb calcium and phosphate ions in saliva [4]. High calcium and phosphate concentrations will increase calcium and phosphate precipitation in microporosity. This precipitation will close the pores on the enamel surface so that the remineralization process of enamel can occur [4]. Other studies have also shown similar results, depth of the lesion formed decreases after being brushed using toothpaste containing hydroxyapatite for 15 days [21]. Hydroxyapatite acts as a source calcium and phosphate ions are needed in the remineralization process and form a thin layer that binds strongly to the tooth surface and prevent the loss of ions when the demineralization process occurs [21,22].

Increased hardness in the sample group is not only affected by the presence of calcium and phosphate content alone, tricalcium phosphate (TCP) contained in hydroxyapatite powder also has a role in increasing enamel hardness. Tricalcium phosphate is a biomaterial that is used for biological applications because it is one of the calcium phosphate minerals found in enamel and bone. Tricalcium phosphate is divided into α TCP and β TCP [23,24]. α TCP has a particle size of 4-10 μm and its structure is monoclinic whereas β TCP has a particle size of 0.5-15 μm and its structure is rhombohedral. α TCP appears when heated at 1400 °C for 2 hours while β TCP appears when heated at temperatures above 800°C [17,23,24]. Based on these data it is suspected that TCP formed in this study is β TCP because the temperature used for calcination is 900 °C. β TCP is soluble and has a smaller particle size compared to α TCP so it will be easier to enter the pores of the enamel and help remineralize the enamel. Besides being soluble, TCP can protect teeth against bacterial initiation and development, reduce hypersensitivity, and provide optimal benefits when given at neutral pH [25]. Post hoc test results in Table 2 show that groups A, B, and C significantly different due to differences concentration of hydroxyapatite paste given. Increasing concentration of hydroxyapatite, the amount of calcium and phosphate produced will increase so that the process of hydroxyapatite crystal formation will be faster [10]. The formation of apatite crystals begins with the deposition of minerals that will form crystals in small size and not perfect in size and structure. Over time, the crystals formed will blend and perfect themselves to form larger crystals until they reach their maximum size, this event is called Ostwald Ripening [10,12]. Precipitation of hydroxyapatite will initially occur in the
enamel surface layer which has demineralized, then over time the deposition will be forwarded to the inside of the email for long-term remineralization [26]. In addition to the groups that are significantly different, there are also groups that are not significantly different in groups A with group D (control). This is presumably because the 5% concentration has not been able to provide a real remineralization effect on the enamel surface which has been carried out by the demineralization process. Therefore, a concentration of 5% cannot be used as remineralization of email.

5. Conclusion
The conclusion of this study is the application of hydroxyapatite paste from tilapia bone waste can increase surface hardness of enamel.

6. References
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