Synthesis and potential of skipjack tuna bone hydroxyapatite as bone tissue engineering biomaterial

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Abstract. Hydroxyapatite Ca₁₀(PO₄)₆(OH)₂ is an alloplast material that is used to increase bone regeneration. It can be synthesized by processing natural materials such as fish bones. The purpose of this study was to synthesize hydroxyapatite from natural resources skipjack tuna (Katsuwonus pelamis) bones with precipitation method. Then, characterize hydroxyapatite morphology with FESEM and its biocompatibility using the preosteoblast MC3T3-E1 cell line. Skipjack tuna bone was synthesized into hydroxyapatite through precipitation method. The morphology of hydroxyapatite sample was revealed with field-emission scanning microscope FESEM. While the constituent elements were analyzed using SEM EDAX. Biocompatibility of hydroxyapatite was tested using preosteoblast cell culture. Cells were treated with different hydroxyapatite concentration 200 µg/ml, 100 µg/ml and 5 µg/ml. After incubation with CO₂ 5% at 37°C for 24h, 48h and 72h the culture was tested for viability using MTT Cell Viability Assay Kit. Results were reported as optical density. The study showed that skipjack tuna bone produced grain-shaped particles with almost uniform sizes. The surface material appears to be agglomerates and form pores in between. Weight percentages Ca/P ratio for hydroxyapatite from skipjack tuna bones is 1.94. MTT assay showed cell viability after 3 days. These results suggest that skipjack tuna bone hydroxyapatite is has potential as bone engineering biomaterial.

1. Introduction
Tooth extraction is a common dental procedure. Several causes of tooth extraction are tooth decay due to caries, pericoronitis, trauma, periodontal disease, orthodontic treatment or impactions [1]. Unfortunately, tooth extraction causes alveolar bone resorption. Most of bone loss will occur within six months after extraction [2]. Bone loss will lead to a reduction in bucolingual and apicocoronal dimensions of alveolar bone. The alveolar ridge undergoes more resorption on buccal side than lingual side [3]. The shortened residual ridge will complicate support and retention of prosthesis. Especially in mandible, which has four times resorption than maxilla [4].
To overcome bone loss and minimize resorption, application of bone graft material is used. The choice of graft material must meet several requirements, such as biocompatibility, osteoconductivity and osteoinductivity. Autograft materials which is using donor from parts of patient's body are ideal materials because of these properties. However, autografts have several disadvantages such as requiring additional surgical procedures, limited donors and high levels of reabsorption [2, 5].

Another graft material that is highly biocompatible but requires a complicated procedure is the allograft. The disadvantage of this material is that it still has to take tissue from other humans and eliminate possibility of an immunogenic response. Meanwhile, xenograft is more capable of mass production at affordable costs. However, it has the potential to induce an immune response and the possibility of disease transmission [6]. To overcome this problem, alloplast materials are developed which are derived from inorganic materials without animal or human components. Therefore, it can reduce potential for disease transmission and reduce donor site morbidity [7].

Hydroxyapatite $\text{Ca}_10(\text{PO}_4)_6(\text{OH})_2$ is an alloplast material that is used to increase bone regeneration [8]. This material is most widely used because of its biocompatibility and osteoconductivity properties [9]. Hydroxyapatite belongs to the calcium phosphate group with a composition similar to inorganic components of natural bone and teeth [6, 10, 11].

Hydroxyapatite synthesis can be done by processing natural materials such as fish bones. The increasing production of fish-based packaged food will certainly produce a lot of waste. Therefore we can use it as a base material for hydroxyapatite [12, 13]. Several previous studies have synthesized hydroxyapatite through calcination, precipitation, alkaline hydrolysis, hydrothermal or combination methods [14, 15]. The bones used also vary, such as rainbow trout, cod, salmon and tuna. The result is a non-toxic hydroxyapatite which is quite promising as a bone tissue engineering biomaterial [8, 12].

The purpose of this study was to synthesize hydroxyapatite from natural ingredients skipjack tuna (*Katsuwonus pelamis*) fish bones with precipitation method. Then, characterize hydroxyapatite morphology with FESEM and its biocompatibility using the MC3T3-E1 cell line. Thus, it can determine potential of skipjack tuna bone hydroxyapatite as bone tissue engineering biomaterial.

### 2. Methods

#### 2.1. Hydroxyapatite synthesis

Skipjack tuna (*Katsuwonus pelamis*) fish bone were supplied by fish processing factory in Pasuruan, Indonesia. The fish bones were cleaned and dried in the sun. After drying, soaked in 98% acetone for 3 days. Then it is dried again until acetone evaporates and aroma is gone. Furthermore, the calcination was carried out in a furnace at a temperature of 900°C for 5 hours. After that, bones were manually crushed into powder and then sieved with a 125mesh sieve to produce CaO powder.

CaO powder was put in a beaker plus 100 ml distilled water, stirred for 1 hour at 90°C using a magnetic stirrer. After 1 hour, 0.6 M phosphoric acid solution was added to burette and stirred again for 1 hour at the same temperature. Then adjust the pH of the solution using 1M sodium hydroxide to pH 10. The solution was deposited for 24 hours, filtered and the precipitate heated for 2 hours at a temperature of 105°C. Then it was heated again in furnace for 5 hours at a temperature of 900°C [16]. The morphology of hydroxyapatite skipjack tuna fish bone sample were revealed with field-emission scanning microscope FESEM (FEI Quanta FEG 650). While the constituent elements were analyzed using SEM EDAX (FEI Inspect S50).

#### 2.2. Cell culture and Viability assay

Mouse preosteoblasts MC3T3-E1 (ATCC, US) was used in present study cultured in Alpha Minimum Essential Medium (Gibco), containing 10% Fetal Bovine Serum (Sigma) and 1% Antibiotic Antimycotic (Gibco) in a humidified atmosphere of 5% CO$_2$ at 37°C.
Viability of preosteoblasts MC3T3-E1 was determined using Cell Quanti-MTT Cell Viability Assay Kit (Bioassay). Cells were seeded in 96-well plates (Corning) at density 0.5 x 10^4 per well. After 70% confluence, the samples of different hydroxyapatite concentration 200 µg/ml, 100 µg/ml and 5 µg/ml was added to the cells. After incubation with CO₂ 5% at 37 °C for 24, 48, and 72h the culture was added reagent kit 18.8 µl per well then incubated for 4 hours at 37°C. Next, 100µl solubilizer added to each well and shake for 1 hour at room temperature. At the end, the absorbance value was determined at 570 nm with microplate reader (SPECTROstar Nano). Each test was performed in triplicate. Results were reported as optical density [12].

2.3. Statistical analysis
Statistics was performed using SPSS 25. All data were gathered in independent quadruplicate and described as the mean values in a chart. The statistical differences were determined by ANOVA test. Significance difference was established at p < 0.05.

3. Result and Discussions
3.1 FESEM analysis
Results of FESEM analysis is showed in Figure 1. FESEM will show shape and surface morphology particles of hydroxyapatite.

![FESEM images of hydroxyapatite skipjack tuna bone magnification 100x.](image)
3.2 SEM EDAX analysis
SEM EDAX analysis showed element composition of hydroxyapatite in Figure 3. This study will count Calcium/Phosphate ratio by its weight percentages in Table 1.

![Figure 3 SEM EDAX Spectra for skipjack tuna bone hydroxyapatite](image)

| Table 1. Calcium/Phosphate ratio (Wt%) |
|---------------------------------------|
| Calcium | 38.77 |
| Phosphorus | 20.02 |
| Ca/P | 1.94 |

3.3 Viability assay
Viability of preosteoblast cell culture MC3T3-E1 showed by its optical density after treated and tested with MTT Assay in Figure 4. It was treated with samples of different hydroxyapatite concentration of 200 µg/ml, 100 µg/ml and 5 µg/ml and incubation for 24, 48 dan 72 hours.
Hydroxyapatite is a calcium phosphate material which is widely used in dentistry. This study discusses hydroxyapatite as bone substitute due to its similarity with natural bone. Some of the specific characteristics of particles that are important are size, shape, surface and morphology. The results of this study showed that skipjack tuna (*Katsuwonus pelamis*) bone hydroxyapatite which was synthesized by precipitation method produced grain-shaped particles with almost uniform sizes (Figure 1, 2). The surface material appears to be agglomerates and form pores in between. These characteristics will affect its ability to regenerate bone. The small and uniform particle size will increase the dissolution rate and bioavailability. A spherical form will have better flowability [17]. The rough and porous particle surface is useful for cell attachment. This characteristics will be useful to increase adhesion and proliferation of osteoblast cells when used as bone regeneration material [18, 19].

Element composition of hydroxyapatite were examined by SEM EDAX [20]. The results showed characteristic peaks of calcium, phosphorus and oxygen hydroxyapatite of skipjack fish bones (Figure 3). This value is considered to provide a Ca / P ratio for hydroxyapatite from skipjack tuna bones, which is 1.94 for weight percentages (Table 1) [21]. It is higher compared to stoichiometric hydroxyapatite (Ca / P = 1.67). The Ca / P ratio shows the solubility of calcium phosphate compounds. The higher the value, the higher the solubility [22]. The higher the solubility, the easier to change the local pH and ion concentration. This will affect protein adhesion which is useful for increasing cell adhesion thus affecting the effectiveness of bone regeneration [23].

The next characteristic is biocompatibility. Figure 4 shows the optical density of MTT assay on preosteoblast cell line MC3T3-E1 against skipjack tuna fish bone hydroxyapatite at 3 different concentrations for 24, 48 and 72 hours. The mtt reagent will bind to the mitochondria of the living cell then the optical density showed cell viability to determine whether the treatment has cell toxicity [24, 25, 26]. Although the optical density did not show a significant difference compared to controls, the results showed increase in cell viability. Best cell viability especially after 72 hours. This result is in accordance with the study of Shi, et al, which also showed significant increase in cell viability after 3 days. Meanwhile, the optimum concentration is 50µg / ml.[12] These results suggest that skipjack tuna fish bone hydroxyapatite is biocompatible. The value of cell viability that was higher than the control also showed that the cells had proliferation [27, 28].

The instability of this cell viability value can be caused by the shape and size of the particles that are less homogeneous. Research by Geng, et al. Showed that the spherical shape will produce favorable properties on osteoblast cells compared to rod likes. A smaller size will also increase solubility. The hydroxyapatite particles that dissolve will release Ca^{2+} ions. The right ion concentration...
will increase the proliferation of preosteoblast cells, but the increase in Ca\(^{2+}\) can also trigger cell apoptosis [29, 30].

4. Conclusion
Skipjack tuna (Katsuwonus pelamis) bone hydroxyapatite which was synthesized by the precipitation method produced grain-shaped particle with Ca/P ratio 1.94. This material is biocompatible. Optimum concentration to stimulate cell proliferation in this study is 50µg / ml after 72 hours. Taken together, these findings showed that skipjack tuna bone has potential as biomaterial source of hydroxyapatite.

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