Extraction and characterization of nano calcium from tilapia (Oreochromis niloticus) scales from Semarang, Indonesia

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Abstract. The research aims to develop a method to extract nano calcium from tilapia (Oreochromis niloticus) scales. Yielding nano calcium from the fish scale will enable the marine and fisheries community to gain a higher economic value from this untapped source. The first study was designed to find the best acid solution (citric acid and HCl) and time (3 and 6 h) for the extraction. The result revealed that calcium extracted with HCl for 3 h had a higher calcium content and yield than other treatments. The following study was conducted to find the best concentration of HCl and time for the extraction. The result revealed that calcium extracted for 30 mins with HCl 0.3 N had calcium content higher than other treatments. The powder is then ground with high energy milling (HEM) at a different time (3 and 2 h, and 15, 30, 35, 60, 75 mins) to get nano-sized particles. The Morphology and elements were analyzed using scanning electron microscopy (SEM) and energy dispersive X-ray (EDX). To conclude, extracting with 0.3 N HCl for 60 mins and with a milling time for 45 mins was the best method to extract nano calcium from tilapia scales. The nano calcium can be used as additional ingredients for snacks to add value.

1. Introduction

In Indonesia, Nile and Mozambique, tilapia production has been growing almost 400%, from 337,221 tones in 2009 to 1,257,000 tones in 2019 [1]. The production leads to significant growth in tilapia processing. Tilapia fillets have become one of the most popular fillets in Indonesia, which a high demand on both domestic and international markets [2]. The development has caused more waste, like viscera, heads, skin, bones [3]. The unmanaged byproduct from the processing can cause environmental problems and lead to health problems for humans.

Research on utilizing the byproduct to create value-added products has been a focus of many scientists in the last decade [3,4,5]. Besides protein, calcium is the most abundant substance in fish waste. Fishbone carries about 60 – 70% mineral of its total mass [6], and 34 – 36% of it is calcium [7], making it the promising source for bio-calcium composting. The knowledge about the extraction technology and characteristic of calcium from fishbone is well provided [5,7,8]. Besides bone, scales also hold many minerals, including calcium (3.32 mg/kg) [9]. However, little information is known regarding the extraction technology, physicochemical characteristics of calcium from scales.

Calcium and its derivatives yielded from natural sources show a high bioavailability with no cellular toxicity and fibrous tissue generation around the healing area [10,11,12,13]. Calcium at nano-sized is more likely to be chosen by the consumers since the body easily absorbs it. According to [14],
the size of nanoparticles should vary between 1 – 1000 nm. The technology around obtaining nano-sized calcium from natural sources originating from Indonesia, such as fishbone, seashells, cow bone, and eggshells, is well established. Meanwhile, there are few published studies on producing nano calcium from fish scales, especially fish from Indonesia waters. This research aims to develop a method to extract nano calcium from tilapia (Oreochromis niloticus) scales and characterize the powder. Producing nano calcium from fish waste will enable the marine and fisheries community to gain a higher economic value of this low-value product. Thus, the research is worth conducting.

2. Material and methods

2.1. Preparation of tilapia scales
Fresh tilapia scales, approximately between ø 0.3 – 0.6 mm, were obtained from a tilapia processing plant in Semarang, Centre Java, Indonesia. The scales were transferred to the laboratory by shipment on ice in iceboxes during transportation. The scales were washed with fresh water in a plastic container to remove dirt and unwanted substances upon arrival. The scales were then rinsed. The clean scales were transferred onto plastic trays and sundried afterward. The dry scales were ready to be used.

2.2. Experimental design
Nano calcium powder was extracted from tilapia using acidic methods. The study involves three experiments. The first experiment was designed to find the best acid solution and time for the extraction. The following experiment was conducted to find the best concentration of the best solvent from experiment 1 and the time for extraction. The last experiment was designed to find the optimum time to grind the powder to get nano-sized particles of nano calcium.

2.2.1. Effect of solvent and time of extraction to scales powder characteristics. Two different solvents (HCl, 0.5 N, Merck 109057) and (citric acid, 2% w/v, Merck 100241) were used to extract calcium from the scales. The extraction was done in a waterbath (Memmert, Schwabach Germany), at 55º C for 3 and 6 h. After extraction, the scales then drained and sundried. The dried scales were ground using a hammer mill until they became a fine powder. Yield then calculated. Moisture and ash analysis, calcium content analysis, and whiteness analysis were conducted.

2.2.2. Effect of solvent concentration and time of extraction on the calcium powder physicochemical characteristics. Three different concentrations of HCl (0.1, 0.3, and 0.5 N) were used in this experiment. The scales were then extracted for 30, 60, and 90 mins in the water bath at 90 °C. After extraction, the scales were drained and soaked in NaOH (Merck 106498) 2 N for 15 minutes. After 15 minutes, the scales were drained and washed. The washed, then sundried until fully dry. The dry scales were ground with the same protocols as mentioned in Section 2.2.1. The powder was milled in high-energy milling (HEM) (BATAN, Serpong, Indonesia) at 1000 rpm for 3 and 2 h to get nano-sized particles. Physicochemical analysis was conducted to characterize the calcium powder.

2.2.3. High energy milling (HEM) optimization. The optimization was done by focusing on milling time (15, 30, 45, 60, 75, and 90 mins) at 1000 rpm using the same machine mentioned in Section 2.2.2.

2.3. Physicochemical characterization methods
The measurement of moisture content and ash content was performed following [15,16]. Calcium content analysis was performed using atomic absorption spectrometry (AAS) (Perkin Elmer Analyst 100 type flame).

Analysis on whiteness was done using ColorFlex EZ (Hunterlab Reston, Virginia, USA). The whiteness measurement was determined based on knowing the value of lightness (L*); green-redness
(a*), where -a* indicates green and +a* indicates red; and blue-yellowness (b*), where -b* indicates blue and +b* indicates yellow. The value is then determined using the below equation:

\[
\text{Whiteness} = 100 - [(100-L*)^2 + (a*)^2 + (b*)^2]^{1/2}
\]  

(1)

The calcium powder and elements morphology was characterized using scanning electron microscopy with energy dispersive X-ray (SEM-EDX) (JSM-35C, JEOL, Tokyo, Japan) at 22 kV. Gold (Au) was used to coat calcium to avoid conductivity during the visualization process.

The size of the powder was analyzed with a particle size analyzer (PSA) (Vasco-PSA, refractometer Arago DL, 135 Corduan, Germany). Prior analysis, calcium was dispersed in distilled water. Samples were measured using low-angle laser light scattering (LALLS), measuring from 0.1 – 3000 µm particles with laser light.

2.4. Statistical analysis

Two-way ANOVA was used to determine the interaction between factors and which factor had a significant effect on experiments 1 and 2. Statistical analysis was performed using Prism9 (GraphPad Prism software version 9.12, CA, USA).

3. Results and discussion

3.1. Effect of solvent and time of extraction on the yield of calcium powder

The effect of solvent and time on the calcium powder needs to be investigated to find the best practice methods of extracting the calcium from the scales. The type of solvent and time can also impact the production effectiveness besides the quality of the calcium. The solvent used for extraction had a significant effect on the moisture content (p< 0.05) (Table 1.). However, the interaction between factors (solvent and time) did not significantly affect moisture content. The same result went for ash content and whiteness that even the solvent gave a significant effect to the ash content (p< 0.05), the interaction between factors was not significant (p>0.05). The ash content of scales powder extracted with HCl is higher than Mozambique tilapia from Lake Lindu 21.85% [9].

On the other hand, the time and interaction between factors significantly affected the calcium content (p< 0.05). From Table 1, the calcium content of scales powder extracted with HCl 0.5 N for 3 h was higher than other treatments and what was reported by [9] 332 mg/100g. The treatments did not have any effect on the yield (p>0.05). There is no publication to compare regarding the yield from extracted calcium from fish scales.

| Parameter                  | Treatment A (HCl 0.5 N, 3 h) | Treatment B (HCl 0.5 N, 6 h) | Treatment C (Citric acid 2%, 3 h) | Treatment D (Citric acid 2%, 6 h) |
|----------------------------|-------------------------------|-----------------------------|----------------------------------|----------------------------------|
| Moisture content (%)       | 11.6 ± 0.0^a                  | 11.5 ± 0.6^a                | 12.5 ± 0.1^b                     | 12.9 ± 0.2^b                     |
| Ash content (%)            | 30.4 ± 0.7^a                  | 32.2± 0.1^a                 | 19.4 ± 0.3^b                     | 19.5 ± 0.1^b                     |
| Calcium content (mg/100g)  | 434.3 ± 2.2^a                 | 223.5 ± 2.2^b              | 323.0 ± 3.2^a                    | 330.4 ± 2.4^b                    |
| Whiteness                  | 74.6 ± 0.1^a                  | 73.2 ± 0.0^a                | 70.4 ± 0.2^b                     | 72 ± 0.9^b                       |
| Yield (%)                  | 57.5 ± 0.1^a                  | 55.5 ± 1.3^a               | 52.8 ± 3.1^a                     | 54.3 ± 1.9^a                     |

Note: superscripts label denotes significant difference across treatments.
The result shows that it is clear that HCl gave a better characteristic to the yielded scales powder. Thus, HCl in the following experiment was used to extract the calcium from tilapia scales with three different concentrations and times.

3.2. Effect of solvent concentration of extraction to the calcium powder physicochemical characteristic

Even though it is reported that a high concentration of NaOH and time give a high calcium content from fishbone [5], the result of this study was the opposite. The result also reveals that a lower concentration of HCl gave a higher level of calcium content. The concentration and time did not have a significant effect on moisture content ($p > 0.05$) (Table 2). However, the time and concentration significantly affected the calcium content ($p < 0.05$). Research on determining the optimum condition of extracting calcium from catfish also proved that time and solvent have a significant effect on the yield of the calcium, the calcium content, and whiteness [17]. The results of their study obtained a linear model used to predict responses. The maximum response value is obtained with 5% NaOH for 30 minutes or 11.64% HCl for 58 minutes. The verification of the treatment obtained the amount of calcium 15.74-17.46% with an accuracy rate of more than 87.5% [17].

Table 2. Physicochemical characteristics of calcium powder for tilapia scales.

| Treatment       | Moisture content (%) (n=4/df=2) | Ash content (%) (n=4/df=2) | Calcium content (mg/100g) (n=4/df=2) | Whiteness (%) (n=4/df=2) | Yield (%) (n=6/df=2) |
|-----------------|---------------------------------|-----------------------------|--------------------------------------|--------------------------|----------------------|
| A1 (0.1 N, 30') | 7.4 ± 0.3a                     | 56.9 ± 1.1a                 | 402. ± 1.7a                          | 88.3 ± 0.1a              | 58.7 ± 0.6a         |
| A2 (0.3 N, 30') | 6.8 ± 0.9a                     | 52.± 16.2b                 | 278.0 ± 0.9b                         | 93.2 ± 0.1b              | 56.2 ± 0.6b         |
| A3 (0.5 N, 30') | 7.9 ± 0.0a                     | 30.5 ± 2.5c                | 301.4 ± 1.0c                         | 83.4 ± 0.1c              | 57.0 ± 0.8c         |
| B1 (0.1 N, 60') | 7.1 ± 0.4d                     | 57.4 ± 1.3d                | 450.6 ± 3.6d                         | 91.5 ± 0.1d              | 57.4 ± 0.8d         |
| B2 (0.3 N, 60') | 6.5 ± 1.2a                     | 61.6 ± 1.3b                | 233.2 ± 1.4c                         | 92.2 ± 0.1c              | 56.0 ± 0.1c         |
| B3 (0.5 N, 60') | 9.7 ± 1.1a                     | 23.9 ± 11.63c              | 225.5 ± 1.1f                         | 87.5 ± 0.1f              | 55.2 ± 0.4d         |
| C1 (0.1 N, 90') | 7.4 ± 0.4d                     | 64.3 ± 2.5a                | 428.1 ± 1.3f                         | 82.8 ± 0.1g              | 57.9 ± 0.7g         |
| C1 (0.3 N, 90') | 6.2 ± 0.1a                     | 63.0 ± 5.6b                | 420.1 ± 1.3b                         | 83.8 ± 0.1h              | 56.2 ± 0.8b         |
| C1 (0.5 N, 90') | 9.1 ± 0.4a                     | 24.4 ± 0.1c                | 273.4 ± 1.5i                         | 97.1 ± 0.1i              | 56.7 ± 0.7i         |

Note: superscripts label denotes significant difference across treatments.

Figure 1. Morphology of calcium powder extracted with HCl 0.1 N with SEM at 400x magnification. (A) morphology of calcium powder extracted for 30 min (A1) s; (B) sample extracted for 60 mins 60 (B1); (C) sample extracted for 90 mins (C1).
Figure 2. Morphology of calcium powder extracted with HCl 0.3 N for 30 mins with SEM at 3000x magnification. (A) after milled with HEM for 2 h; (B) after milled with HEM for 3 h.

The morphological analysis shows that calcium powder resulting from different extraction times has a similar form. According to the analysis, the powder was not evenly distributed and had a similar size (Figure 1). Figure 1 also showed that the surface of all samples was smooth, with no pores detected. The pores have a significant effect on bone tissue engineering [18]. They all have pointy shapes. However, the effect of the pointy shape of calcium powder on the quality and safety is not well documented yet. The morphology of calcium powder milled with HEM became finer micro-sized (Figure 2). In addition, the figure also shows that agglomeration seems to happen to all samples. It is crucial to avoid the inviting forces between particles during the milling process because it produces heat from the interaction between balls and particles. The solid and thick cooperatives of nanoparticles signify the aggregation, yet the freely joint particles show the agglomeration, which might be broken by mechanical pressure (19).

The elemental analysis using EDX showed that the calcium and oxygen content level was increased after the powder was milled with HEM for 3 h. The air may have caused the high level of oxygen during the milling process. It also showed that the calcium that yielded might be calcium oxide (CaO).

Table 3. Elemental analysis using EDX of calcium powder obtained from extraction with HCl 0.5 N for 30 minutes, n = 2.

| Element  | Before HEM | After HEM (3 h) |
|----------|------------|-----------------|
| Carbon (C) | 20.9 ± 0.6 | 15.7 ± 0.9 |
| Nitrogen (N) | 12.9 ± 6.5 | 5.5 ± 2.2 |
| Oxygen (O) | 38.0 ± 9.2 | 42.0 ± 2.2 |
| Natrium (Na) | 0.9 ± 0.3 | 1.31 ± 0.1 |
| Phosphor (P) | 8.6 ± 1.1 | 10.1 ± 0.1 |
| Calcium (Ca) | 19.2 ± 5.8 | 25.5 ± 0.8 |

3.3. HEM optimization
The best range size of nanoparticles to be effective as drug delivery is between 200 – 400 nm [20]. Even though milled time gave nano-sized particles of calcium, it seems that milling the calcium powder with HEM at 1000 rpm for 15 - 45 minutes seems to be the best practice to avoid agglomeration (Table 4). Agglomeration of nanoparticles diminishes the likely improvement of mechanical properties in nanocomposites because of the limitation of the interfacial region [21]. Therefore, finding appropriate milling time with HEM is a major challenge. The size of nano calcium powder from this research was smaller than calcium that was extracted from tilapia bone (Oreochromis niloticus) soaking on Averrhoa bilimbi, which was the size were between 1,158 to 4,455.
nm [22]. The acid concentration from *Averrhoa bilimbi* is presumably lower than HCl, thus it can not produce a smaller size of the powder.

### Table 4. The size of nano calcium after milling with HEM for certain minutes.

| Mins | Size (nm)    |
|------|-------------|
| 15   | 219.5 ± 0.7^a |
| 30   | 275.9 ± 1.6^b |
| 45   | 195.5 ± 1.6^c |
| 60   | 228.7 ± 0.9^d |
| 75   | 231.7 ± 2.2^e |
| 90   | 214.0 ± 2.7^f |

A further study using water (H$_2$O) to avoid agglomeration during the milling may be worth conducting. The previous study [20,23] showed that the agglomeration rate of calcium hydroxide (Ca(OH)$_2$) particles was faster than that of CaO particles in the presence of H$_2$O, which was attributed to the greater spatial displacements of atoms in the reactant particles when the thermochemical reaction occurred.

### 4. Conclusions

In overall conclusion, the study has successfully extracted calcium from tilapia scales. Time and type of solvent were affecting the characteristics of the calcium powder. The nano calcium was successfully obtained using HEM. Extracting with 0.3 N HCl for 60 mins and with a milling time of 45 mins was the best method to extract nano calcium from tilapia scales. The size of yielded calcium from the best treatment was 195.5 nm. The nano calcium can be used as a food ingredient on a snack to add value. It is suggested to conduct a further experiment on the mechanism of avoiding agglomeration, calcium solubility, and biocompatibility.

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