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Production of dicalcium phosphate anhydrous from the bone of Nile Tilapia (*Oreochromis niloticus)* grown in Viet Nam

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ABSTRACT

This study was conducted to find a facile method for preparing dicalcium phosphate anhydrous (DCPA) from the bones of Nile Tilapia, recovered from a by-product of the fillet process. The by-product containing bones of the Nile Tilapia collected from a local factory was stewed in boiling water and treated with NaOH solution to remove organic impurities from the obtained bones of Tilapia. The pre-treated bones were then calcined and ground to a fine powder before converting to dicalcium phosphate anhydrous. The factors affecting the yield of DCPA such as precipitation pH, reaction temperature, and reaction time were investigated. Modern analytical methods, such as XRD, SEM, XRF, and TGA were employed to characterize the DCPA. The DCPA product with high crystallinity (~ 98.5%) with particle sizes ranging from 1 to 3 µm was obtained at pH 5, 90 min. reaction, and 80°C. This study showed a facile and viable method for producing dicalcium phosphate anhydrous from the bones of Nile Tilapia.

1. INTRODUCTION

Dicalcium phosphate ($CaHPO_4$) is a type of calcium phosphate mineral that exists in two crystalline forms: dicalcium phosphate dihydrate (DCPD, brushite) and dicalcium phosphate anhydrous (DCPA, monetite) (Zhou et al., 2021). The anhydrous form has higher porosity due to the absence of two water molecules in the crystal structure, which can lead to better compressibility and faster disintegration. DCPA is considered an osteoconductive biomaterial with high potential as a bio-resorbable cement for bone regeneration (Tamimi et al., 2012; Gou et al., 2024). This material can be used to reconstruct hard tissues because of its better resorption compared to calcium hydroxyapatite, octacalcium phosphate, and tricalcium phosphate, with higher solubility in vivo of monetite (Eshtiagh-Hosseini et al., 2008). Pure DCPA is rare in nature because it mainly exists as an accompanying component with other materials. Therefore, studying to find a good method to synthesize DCPA from natural calcium resources was interesting to scientists.

DCPA can be synthesized using different pure chemicals containing calcium and phosphorus ions through various methods (Motameni, Alshemary, & Evis, 2021). For example, DCPA can be prepared through a solid phase reaction by combining calcium and phosphorus precursors with a Ca/P ratio of 1 (Duncan et al., 2014). It can also be created by neutralizing phosphoric acid with calcium hydroxide in aqueous solutions at a pH of 3-4 and mild temperature (60°C). Furthermore, DCPA can be obtained by the thermal decomposition of DCPD

at high temperatures of 200-220°C (Mulongo-Masamba et al., 2016; Zhou et al., 2021). Additionally, DCPA derived from natural calcium resources such as eggshells, animal bones, and fish bones has shown highly absorbable behaviors, superior to pure chemicals due to trace element contribution (Huy & Thanh, 2016).

In Vietnam, there has been a rapid increase in tilapia production in recent years, resulting in the generation of solid waste during fish fillet processing, which mainly consists of bones, skin, and scales. Finding a valuable use for this solid waste can be beneficial. Currently, fish bones are used as animal feed and in various industries as calcium supplements, but their value is not very high. This study aims to use tilapia bones to produce DCPA, a substance with potential applications in biomedical engineering and bone tissue.

2. MATERIALS AND METHOD

2.1. Chemicals and Materials

The by-product of Nile Tilapia containing bones was collected from a factory in the Tra Noc industrial zone, Can Tho City, Vietnam, and kept at -30°C for later use. The chemicals used in this study included sodium hydroxide (NaOH), ethanol (C_2H_5OH), hydrochloric acid (HCl), and ammonia solution (NH_4OH) which were purchased from Sigma-Aldrich USA.

2.2. Preparation of DCPA from Nile Tilapia bone

In order to produce DCPA, the by-product of Nile Tilapia, which contains bones, was boiled for 2 hours and then treated with 0.1 N NaOH solution (1:2 $g.mL^{-1}$) for 12 hours to remove organic impurities and obtain pure bones. The bones were calcined at 600°C for 6 hours in a Nabertherm furnace and then ground to fine fish bone powder (FBP) using a high-speed ball mill (Model SD/2-1000, Ceramic Instrument, Italy). The FBP was then stored in desiccators to prevent water adsorption from the atmosphere. Subsequently, the FBP reacted with 0.5 N hydrochloric acid (HCl) at a solid-to-liquid ratio of 1:15 $g.mL^{-1}$ in an aqueous solution at 30°C for 1 hour using a magnetic stirrer at 400 rpm. After the filtration process, the pH of the filtrate was adjusted from 4 to 7 by slowly adding a 25% ammonium hydroxide solution. The mixture was continuously stirred at a temperature ranging from 50 to 80°C for 30 to 120 minutes. The collected precipitate was washed several times with distilled water and then dried at 105°C for 24 hours to obtain the final product. Various important factors affecting the reaction yield of DCPA including precipitation pH, reaction temperature, and reaction time, were thoroughly examined. The DCPA yield was calculated using the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of DCPA product}}{\text{Weight of FBP}} \times 100\%$$

2.3. Characterization methods

X-ray fluorescence spectroscopy: The chemical composition of FBP and DCPA was analyzed by XRF (Epsilon, PAN analytical, USA). These working conditions: emission energy source 50 W, probe resolution 149 Ev, emission dose 0.2 $Sv.h^{-1}$.

X-ray diffraction: DCPA characterizations were determined by XRD, using a D8 Advance from Bruker (Germany) diffractometer in parallel beam geometry equipped with CuKα radiation (wavelength 1.5406 Å). The XRD patterns were collected at a speed of 2°/min and a resolution of 0.02°. The crystal size and crystallinity were calculated based on XRD patterns using the Scherrer formula.

Scanning electron microscopy: The morphology of DCPA was observed by an SEM (HITACHI S-4800, Japan), with magnification from x5 to x40000 with an acceleration voltage of 0.5-30 kV. The size of DCPA particles was determined by image software through SEM images.

Thermo-gravimetric analysis (TGA): The analysis was performed on DCPA powder, using a TG 209 F3 Tarsus® (Deutschland) under a nitrogen atmosphere. The temperature range was varied from room temperature to 1000°C at a heating rate of 10 K/min.

Production yields were statistically analyzed by performing a one-way analysis of variance (ANOVA), executed by Origin software®. Fisher's pairwise comparison test was applied to compare production yields on different samples for $p < 0.05$ or $p < 0.01$.

3. RESULTS AND DISCUSSION

After pretreatment, the results indicated that approximately 11.1% of fish bone by-products were obtained as dry bones, and 6.9% of the by-products consisted of fish bone powder. The chemical composition of the FBP was analyzed by X-ray diffraction.

Table 1. Chemical composition of powder of Tilapia bones

NO.	ELEMENTS	% WEIGHT
1	$CACO_3$	2.17
2	$MGCO_3$	2.70
3	FEO(OH)	1.06
4	$AL_2SI_2O_7$	0.22
5	FES_2	0.01
6	$KALSI_3O_8$	0.67
7	$CA_{10}(PO_4)_6(OH)_2$	92.15
8	OTHER	1.02

The analysis revealed that FBP is mainly composed of the apatite mineral (hydroxyapatite), accounting for 92.15% of the sample, along with small amounts of minerals containing magnesium, aluminum, potassium, and sodium ions. This finding aligns with previous research. FBP contains not only calcium and phosphorus elements, but also other important mineral elements (Fara et al., 2015; Da Cruz et al., 2020; Khamkongkaeo et al., 2021).

Based on the chemical composition of FBP, the chemical reactions involved in synthesizing DCPA could be described using equations (Ardhaoui et al., 2005):

$$Ca_{10}(PO_4)_6(OH)_2 + 20HCl \rightarrow 10CaCl_2 + 6H_3PO_4 + 2H_2O \quad (1)$$

$$H_3PO_4 \leftrightharpoons H_2PO_4^- + H^+ \quad (2)$$

$$H_2PO_4^- \leftrightharpoons HPO_4^{2-} + H^+ \quad (3)$$

$$CaCl_2 \rightarrow Ca^{2+} + 2Cl^- \quad (4)$$

$$NH_4OH \leftrightharpoons NH_4^+ + OH^- \quad (5)$$

$$H^+ + OH^- \leftrightharpoons H_2O \quad (6)$$

$$Ca^{2+} + H_2PO_4^- + 2H_2O \rightarrow CaHPO_4.2H_2O \downarrow + H^+ \quad (7)$$

$$Ca^{2+} + H_2PO_4^- \rightarrow CaHPO_4\downarrow + H^+ \quad (8)$$

$$CaHPO_4.2H_2O \rightarrow CaHPO_4\downarrow + 2H_2O \quad (9)$$

The chemical reactions that formation of DCPA precipitate through just doing modifications related to pH medium, time reaction, and temperature reaction. Following the initial dissolution of the reagents, the solution experiences a pH jump as a result of the endothermic precipitation of monetite crystals (Motameni et al., 2021).

3.1. Effect of precipitation pH on DCPA formation

The experiment investigated the effect of changing the precipitation pH from 4 to 7 on the formation of DCPA. The fixed conditions were at a reaction temperature of 80°C and a reaction time of 90 minutes. Results indicated that the yield of DCPA increased from 66.4% to 78.8% as the precipitation pH was raised from 4 to 6. However, there was an insignificant change in the DCPA yield at higher precipitation pH levels (Table 2). This can be attributed to the presence of other calcium orthophosphate precipitates with larger molecular weight and higher Ca/P ratios than DCPA, which has a Ca/P ratio of 1 (Dorozhkin, 2017) (Zhou et al., 2021).

Table 2. DCPA products synthesized at different precipitation pH

	Precipitation pH			
	4	**5**	**6**	**7**
Yield, %	66.4±0.4	71.8±0.6	78.8±0.3	81.7±0.5
Crystal size, nm	101.5	102.5	92.1	88.5
Crystallinity, %	97.3	98.5	95.3	94.3

Different **superscripts indicate significant differences ($p < 0.05$)*

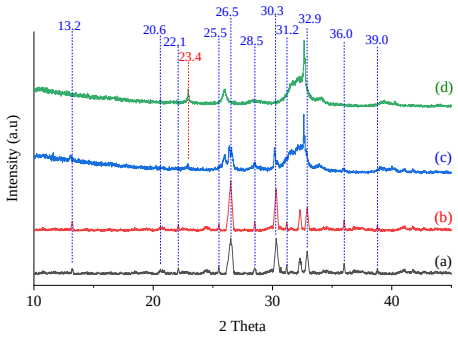

Figure 1. XRD diffractions of DCPA were prepared in different precipitation pH values of (a) pH 4, (b) pH 5, (c) pH 6 and (d) pH 7

For DCPA, the characteristic diffraction peaks shown at 2θ of 13.2°; 26.5°; 28.5°; 30.3°; 32.3°; 32.9°; 36°; 41.5° and 49.5° accordingly with (001), (002), (112), (120), (121), (202), (022), (003) and (440), respectively. These results were consistent with the standard tag (JCPDS No.09-0080). The intensity of DCPA peaks increased with the precipitation pH value rising from pH 4 to pH 5 (Figure 1. a-b). However, at pH 6 and pH 7, XRD shows characteristic peaks of calcium orthophosphate (OCP) at 23.4° 2θ (JCPDS 44-0778) (Figure 1. c-d). The results indicated that the crystallinity of DCPA increased from 97.3% to 98.5% as the pH increased from 4 to 5, but it slightly

decreased at pH 6 and 7. Additionally, the crystal size of DCPA decreased from 101.5 nm to 88.5 nm as the precipitation pH increased from 4 to 7. This suggests that a precipitation pH of 5 is suitable for DCPA synthesis.

3.2. Effect of reaction temperature on DCPA formation

The study investigated by applying temperatures ranging from 50°C to 80°C. The fixed conditions were at pH 5 and a reaction time of 90 minutes. The experimental results demonstrated a slight decrease in DCPA conversion yield, from 83.9% to 71.8%, when the reaction temperature was increased from 50°C to 80°C (Table 3). Furthermore, the intensity of DCPA peaks increased with higher reaction temperatures between 50°C and 80°C (Figure 2). The results also demonstrate that the crystallinity of DCPA increased from 95.6% to 98.5% as the reaction temperature rose from 50°C to 80°C. Additionally, the crystal size of DCPA grew from 89.7 nm to 102.5 nm with the increase in reaction temperature from 50°C to 80°C (Table 3).

Table 3. DCPA products synthesized at different reaction temperature

	Reaction temperature, °C			
	50	**60**	**70**	**80**
Yield, %	83.9±0.5	77.3±0.2	72.8±0.8	71.8±0.3
Crystal size, nm	98.7	99.5	101.4	102.5
Crystallinity, %	95.6	96.4	97.8	98.5

**Different superscripts indicate significant differences ($p < 0.05$)*

The intensity of DCPA peaks gradually increased with rising reaction temperature. Meanwhile, the characteristic peaks of DCPD at positions 2θ of 11.6°; 20.6°; 23.6°; and 29.3° steadily decreased and completely disappeared at 80°C. In the temperature range of 50-80°C, DCPD transforms towards the formation of DCPA. The findings align with previous research indicating that the formation of DCPD releases heat (exothermic), while the formation of DCPA absorbs heat (endothermic). This suggests that DCPD is the initial stage of the precipitation process (Tamimi et al., 2012). The yield of the reaction decreased when the temperature increased from 50°C to 80°C. This is because DCPD formed instead of DCPA at 80°C, which is the optimal temperature for converting DCPA. This implies that a temperature of 80°C is ideal for synthesizing DCPA.

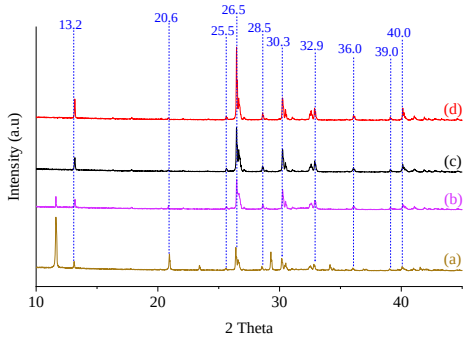

Figure 2. XRD diffractions of DCPA were prepared in different reaction temperatures of (a) 50°C, (b) 60°C, (c) 70°C and (d) 80°C

3.3. Effect of reaction time on DCPA formation

The study examined how the reaction time (ranging from 30 minutes to 120 minutes) affected the formation of DCPA. The fixed conditions were a pH of 5 and a reaction temperature of 80°C. It was observed that the DCPA yield increased slightly from 66.7% to 71.8% as the reaction time was extended from 30 minutes to 90 minutes. However, the yield remained relatively unchanged at longer reaction times (Table 4).

Table 4. DCPA products synthesized at different reaction time

	Reaction time, minutes			
	30	**60**	**90**	**120**
Yield, %	66.7 ±0.3	69.5 ±0.5	71.8 ±0.2	71.9 ±0.3
Crystal size, nm	85.2	98.6	102.5	102.5
Crystallinity, %	98.1	98.3	98.5	98.5

**Different superscripts indicate significant differences ($p < 0.05$)*

For DCPA, the characteristic diffraction peaks shown at 2θ of 13.2°; 26.5°; 28.5°; 30.3°; 32.3°; 32.9°; 36° and 41.5° accordingly with (001), (002), (112), (120), (121), (202), (022) and (003), respectively (JCPDS No.09-0080). Moreover, the intensity of the peaks for DCPA increased as the reaction time rose from 30 minutes to 60 minutes (Figure 3. a-b). The results indicated that the crystallinity of DCPA increased from 98.1% to 98.5% as the reaction time was extended from 30

minutes to 120 minutes. Additionally, the crystal size of DCPA grew from 85.2 nm to 102.5 nm as the reaction time was extended from 30 minutes to 120 minutes (Table 4).

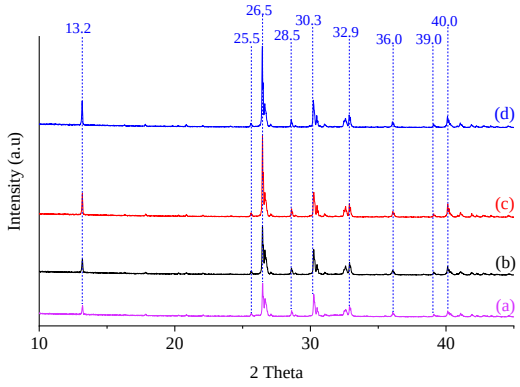

Figure 3. XRD diffractions of DCPA were prepared in different reaction time of (a) 30 min, (b) 60 min, (c) 90 min and (d) 120 min

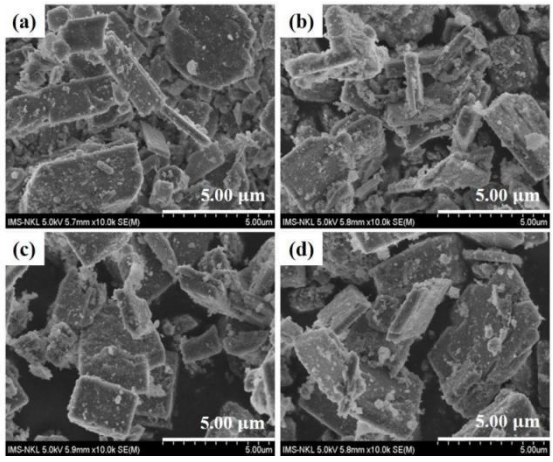

Figure 4. SEM image of DCPA were prepared in different reaction time of (a) 30 min, (b) 60 min, (c) 90 min and (d) 120 min

The SEM analysis revealed that the DCPA powder has a plate-like crystal morphology, as shown in Figure 4. The DCPA particles are 4-6 µm in length, 2-4 µm in width, and roughly 0.5-1.0 µm in thickness. Changing the reaction time from 90 minutes to 120 minutes did not significantly alter the shape, size, or crystallinity of the DCPA particles.

In addition to crystal size and crystallinity gradually increasing from 30 to 90 minutes and insignificantly changing between 90 and 120 minutes, the crystal morphology of powder did not particularly clearly change as extended reaction time from 90 minutes to 120 minutes. Therefore, a reaction time of 90 minutes was deemed suitable for the production of DCPA.

3.4. The characteristics of DCPA product

According to the TGA analysis, no mass loss was observed as the temperature ranged from 30°C to 400°C. However, when the temperature increased to 800°C, the sample experienced a total weight loss of about 12% (Figure 5). This weight loss occurred due to the thermal conversion of DCPA into calcium pyrophosphate during calcination, as the chemical reaction follows: $2CaHPO_4 \rightarrow Ca_2P_2O_7 + H_2O$. These findings are consistent with previous studies had suggested that calcium pyrophosphate powder can be synthesized by thermal conversion of dicalcium phosphate synthesized by precipitation from solutions after the heat treatment at different temperatures in the range of 400-750°C (Dosen & Giese, 2011; Mulongo-Masamba et al., 2016; Sana & Mohamed, 2017).

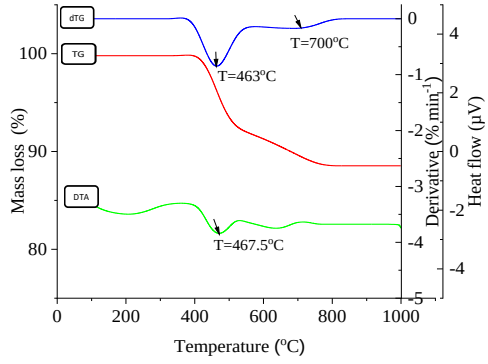

Figure 5. TGA diagram of dicalcium phosphate anhydrous (DCPA) sample synthesized

In this research on the synthesis of dicalcium phosphate from fish bone after the raw material was calcined, not only the raw material was analyzed by XRF first to determine the composition of the fish bone powder, but also DCPA was evaluated for the oxide composition. Based on the results of X-ray fluorescence analysis (Table 5), the DCPA sample included magnesium oxide, iron oxide, and zinc oxide, etc. existed as minor components among the major components (calcium, phosphorus). Dicalcium phosphate with trace elements can promote cell growth, and bone generation and enhance the biocompatibility of materials.

Table 5. XRF analysis of FBP and DCPA

Samples	Chemical composition, %					
	CaO	P_2O_5	MgO	Fe_2O_3	ZnO	other
FBP	67.0	31.2	0.8	0.1	0.06	0.84
DCPA	49.5	50.0	0.1	0.06	0.03	0.31

4. CONCLUSIONS

This study demonstrated an effective method for producing DCPA from Nile Tilapia bones, a by-product of the fillet process. The important parameters, such as pH, reaction temperature, and reaction time, that affected DCPA formation were investigated. DCPA in plate-like crystals with high crystallinity (~98.5%) and particle sizes ranging from 1 to 3 μm were obtained with high reaction yield under specific reaction conditions: pH 5, 90 minutes of reaction time, and 80°C. The successful production of DCPA from fish bones enhances the value of by-products and presents potential applications in the medical field, such as in calcium supplements or biomedical materials.

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