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Physical and chemical characteristics of Asian sea bass bio-calcium powders as affected by ultrasonication treatment and drying method

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Abstract

The effects of ultrasonication and drying method on particle size and other product characteristics of bio-calcium powder from Asian sea bass (*Lates calcarifer*) backbone were investigated. Ultrasonication was performed at different amplitudes (60%, 70%, and 80%) for varying periods (15 and 30 min). Ultrasonication at higher amplitudes for a longer time reduced the powder particle size more effectively ($p < .05$), but had no impact on zeta potential ($p > .05$). The bio-calcium powder ultrasonicated at 70% amplitude for 15 min had the smallest particle size (3.38 μm) when compared to the control (28.85 μm). When the ultrasonicated bio-calcium was subjected to drying, freeze-drying produced powders with higher calcium solubility but lower whiteness than hot air (tray) drying. The results suggest that the ultrasonication is a potential suitable method to reduce the size of bio-calcium powders, while the drying method slightly affected the product characteristics. The bio-calcium powder could serve as a suitable functional ingredient for food fortification aimed at improving the calcium bioavailability.

Particle size of bio-calcium powder from fishbone could affect the mouth feel and calcium solubility when used for food product fortification. This work showed that ultrasonication could be used to obtain up to 10-fold reduction in the particle size of fishbone bio-calcium powders, which promotes increased calcium solubility when subjected to simulated gastrointestinal tract digestion. Few differences in characteristics of the bio-calcium powder were observed for freeze-dried and hot air-dried samples. Thus, an economical, safe, and fast process can be implemented for the production of small particle size bio-calcium powder from fishbone.

KEYWORDS

Asian sea bass, bio-calcium, bone, calcium solubility, drying methods, particle size, ultrasonication

1 | INTRODUCTION

Calcium is an important element that plays a critical role in bone growth and strength, as well as being responsible for the regulation of several cellular and tissue functions such as muscle contraction and exocytosis (Allen et al., 2006). Calcium-fortified products would be useful in improving calcium intake, especially for people with lactose intolerance who cannot consume milk or dairy products (Nemati

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et al., 2017). Previous works have reported that fishbone bio-calcium powder contains 26%–30% of calcium content (Benjakul, Mad-Ali, & Sookchoo, 2017; Benjakul, Mad-Ali, Shenphan, & Sookchoo, 2017; Idowu et al., 2020). This fishbone powder can be used to fortify several foods to increase, not only calcium content but also the level of other minerals such as phosphorous, iron, magnesium, etc. A smaller particle size of bio-calcium powder is required to improve the sensory and palatability of fortified food products. Fortification of calcium salt with a mean particle size ranging from 30 nm to 5 μm for liquid foods such as creamed honey, could reduce after-taste and gritty mouth-feel problems (Wright et al., 2009). Milici et al. (2006) found that calcium salt with particle size reduced to 0.5–5 μm could be readily suspended in syrup and had no harsh off-notes or bitter taste. Cooked cereal dough products could be fortified with calcium phosphate salt with particle sizes of 2–15 μm (Ballman et al., 2001). A previous study showed that fishbone bio-calcium powder had a mean particle size in the range of 10–30 μm (Benjakul et al., 2017; Benjakul, Mad-Ali, & Sookchoo, 2017; Idowu et al., 2020), which may not be well tolerated in food products. Therefore, reduced particle sizes are needed to facilitate the use of bio-calcium powder for food product fortification.

Ultrasonication is identified by the production of acoustic waves, which require a medium to distribute, in which the characteristic nature of the wave can be achieved and further propagated. High energy ultrasound (frequency > 20 kHz) is caused by wave propagation via matters of different properties, leading to compression and decompression of the propagation agents, which produce the acoustic cavitation phenomenon. This generates high pressures and temperatures in the medium, resulting in the formation of bubbles (Cabrera-Trujillo et al., 2016). Ultrasonic cavitation is a physical phenomenon, whose outcome depends upon several parameters such as intensity, frequency, solvent, external pressure, bubbled gas, and temperature, etc. (Santos et al., 2009). This cavitation effect of ultrasound could be used for reducing the particle size of organic substances (Bi et al., 2015; Gilca et al., 2015; Sumari et al., 2013) or inorganic substances (Franco et al., 2004; Hassan et al., 2013; Sompech et al., 2012).

Ultrasound waves can be produced by direct sonication using a transducer horn (ultrasound probe) into the suspension, or by indirect sonication, in which the sample vessel is immersed into a liquid with propagating ultrasonic waves (ultrasound bath) (Taurozzi et al., 2011). After reducing the particle size of bio-calcium by direct sonication in the medium, a drying process is still required for the ease of storage and transportation of resulting product. Different drying methods could have varying influence on characteristics of the ultrasonicated bio-calcium powder. Particle size of bio-calcium powder from fishbone might affect the mouth feel and calcium solubility when being fortified in food products. Differences in drying methods such as freeze-drying and hot air drying might affect the characteristics of final powders. Thus, an economical, safe, and fast process should be developed to obtain small particle size bio-calcium powder from fishbone, in which wider application can be achieved. This study aimed to elucidate the effect of ultrasonication conditions and different drying methods on the particle size and chemical characteristic of the bio-calcium powder produced from Asian sea bass (*Lates calcarifer*) backbone.

2 | MATERIALS AND METHODS

2.1 | Materials/chemicals

2.1.1 | Raw materials

Frozen Asian sea bass frames were purchased from Kingfisher Holdings Co., Ltd., Songkhla, Thailand. Frames were packed with ice in a polystyrene box and transported to the Seafood Laboratory, Prince of Songkhla University, Hat Yai, Thailand within 1 hr. Frames were cut longitudinally into half and furthermore cut to a length of 4–5 cm using a sawing machine (Model W210E, Union Kitchen & Service, Bangkok, Thailand). Prepared bones were washed using tap water until blood and bone marrow were totally removed. The clean bones were kept in the polyethylene bag and stored at −20°C.

2.1.2 | Chemicals

All chemicals for the proximate analysis were purchased from Merck Germany. Hydroxyproline standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 4-Dimethylaminobenzaldehyde was purchased from Ajax, Finechem Pty, Ltd (Auckland, New Zealand). Isopropanol was procured from JT Baker Avantor (PA, USA). Hexane and hydrogen peroxide were purchased from Labscan (RCI Labscan Ltd., Bangkok, Thailand) and QReC (QReC Chemical Co., Ltd, Auckland, New Zealand), respectively.

2.2 | Methods

2.2.1 | Bio-calcium powder preparation

Bio-calcium powder was prepared as per the method of Wijayanti et al. (2020). Cleaned bones were boiled at 100°C using a bone/water ratio of 1:4 (wt/vol) for 30 min to remove adherent meat, then autoclaved at 121°C for 90 min to soften the bone, and dried at 50°C for 24 hr using a hot air oven (Memmert UF30, GmbH + Co. K, Schwabach, Germany). The dry autoclaved bones were ground to coarse powder and soaked in 10 volumes of hexane followed by stirring at 150 rpm using an overhead stirrer (Model RW20.n, IKA-Werke Gmb H&CO. KG, Staufen, Germany) for 60 min at room temperature (RT, 28–30°C), in which fat could be removed. Hexane was drained and samples were left at RT under a fume hood, until hexane smell disappeared and dried. Subsequently, the de-fatted coarse powder was bleached with 2.5% (vol/vol) of hydrogen peroxide using sample-to-solvent ratio of 1:10 (wt/vol), followed by stirring at RT for 60 min. The bleached bones

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were rinsed with tap water for 10 min and dried at 50°C in an oven for 24 hr. The dry coarse powder (100 g) was ground using the ball mill machine (PM 100, Retsch GmbH, Haan, Germany) with 12 grinding balls (20 mm diameter) at 200 rpm for 2.5 hr. The powders were screened by a sieving machine (EVS1, Endecotts Ltd, London, England) using a 75 μm sieve to obtain a fine and uniform powder.

2.2.2 | Ultrasonication of bio-calcium powder

Before the ultrasonication process, bio-calcium powder was added to distilled water at a ratio of 1:4 (wt/vol) to obtain a suspension, which was then ultrasonicated using an ultrasonic device (Model CV 334, Sonics & Material, Inc, Newtown, CT, USA) at 20 kHz ± 50 Hz with intensity power of 750 W under varying conditions. The ultrasonication protocols were performed at 60% amplitude for 15 min (A60T15), 60% amplitude for 30 min (A60T30), 70% amplitude for 15 min (A70T15), 70% amplitude for 30 min (A70T30), 80% amplitude for 15 min (A80T15), and 80% amplitude for 30 min (A80T30) to obtain the ultrasonicated suspensions.

2.2.3 | Particle size analysis

Distribution and mean particle size (D_{43}) of ultrasonicated bio-calcium powder suspensions were determined. A 10 ml aliquot of each suspension was mixed with 5 ml of distilled water prior to analysis by a Laser Particle Size Analyzer (LPSA, LS 230, Coulter, CA, USA).

2.2.4 | Zeta Potential

Zeta potential of ultrasonicated suspensions was analyzed by Zeta Potential Analyzer (Brookhaven Instruments Corp., Holtsville, NY, USA). All the bio-calcium powder suspensions were adjusted to pH 7.2 prior to Zeta potential analysis.

2.2.5 | pH

pH of ultrasonicated suspensions was determined using a pH meter (SI analytical, Scientific Promotion Co Ltd, Bangkok, Thailand).

2.3 | Drying of ultrasonicated bio-calcium with different methods

Control sample was the bio-calcium powder without ultrasonication treatment, which was dried in an oven dryer. The ultrasonicated suspension with the smallest particle size was dried using a freeze dryer (Cool Safe 55, ScanLaf A/S, Lynge, Denmark) at −50°C for 36 hr or a rotary tray dryer at 50°C for 6 hr to obtain powders, which were named BUF and BUR, respectively. The dried samples were further ground using a planetary ball milling with 60 balls (10 mm diameter) at 200 rpm for another 1 hr and were analyzed for chemical and physical characteristics.

2.3.1 | Proximate analysis

Proximate composition of bio-calcium powder was determined according to the AOAC method (AOAC, 2000) for moisture, protein, fat, and ash contents using analytical Nos. 928.08, 950.46, 920.153, and 960.39, respectively.

2.3.2 | Calcium and phosphorus analysis

Calcium and phosphorus contents were determined by an inductively coupled plasma optical emission spectrometer (ICP-OES) (Avio500, Perkin Elmer Instruments, MA, USA). The sample was diluted 100 times and digested by a microwave digester using nitric acid before analysis.

2.3.3 | The solubility of bio-calcium in simulated gastrointestinal tract system

The simulated gastrointestinal tract model system (GIMs) was adopted following the method of Karnjanapratum and Benjakul (2015). The samples (75 mg) were subjected to stomach condition using 50 ml of 5 mM HCl-KCl buffer (pH 1.5) with 2.5 ml of pepsin solution (0.04 g/ml) in 1 of M HCl-KCl buffer (pH 1.5) for 1 hr at 37°C. Furthermore, the reaction was followed by a duodenal condition adjusted to 6.8 with 1 M of $NaHCO_3$. Pancreatic juice containing pancreatin (0.08 g/ml), and bile extract (0.04 g/ml) in 10 mM of Tris–HCl buffer (pH 8.2) were transferred into the mixture and digested for 3 hr. The discontinuation of digestion was done by boiling the sample for 10 min. Later, the digested sample was centrifuged at 7,000 ×*g* for 10 min and the supernatants were taken for calcium analysis by ICP-OES as described above. Bioavailability of calcium was expressed as % relative to the total amount of calcium in the initial (non-sonicated) sample.

2.3.4 | Color

Color of both BUF and BUR was examined by a Hunter lab colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). Whiteness index was calculated according to the equation shown below (Li & Lee, 1996):

$$\text{Whitness Index} = 100 - \sqrt{(100 - L*)^2 + (a*)^2 + (b*)^2}$$

where L^*, a^*, and b^*-values represent lightness, redness/greenness, and yellowness/blueness, respectively. The total difference in color (ΔE^*) was also measured as per the following equation:

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$$\Delta E* = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2}$$

where ΔL^*, Δa^*, and Δb^* are the differences between the corresponding color parameter of the sample and that of white standard ($L^* = 93.63$, $a^* = -0.94$, and $b^* = 0.40$).

2.3.5 | X-ray diffraction (XRD)

X-ray diffraction (XRD) analyses of bio-calcium were done as described by Benjakul, Mad-Ali, Senphan, et al. (2017) using an X-ray Diffractometer (WI-RES-XRD EMPREYAN-001, PANalytical, Netherlands). The wavelength used was 0.154 nm (Cu K-α radiation). The bio-calcium powders were scanned at a 2θ angle ranging from 5° to 90° with the step size of 0.026°at 40 kV, 30 mA, and the time/step of 0.27 s. Phase identification was performed using a peak profile, matching to the reference data from the International Centre for Diffraction Data (ICDD). Degree of crystallinity was measured using the equation shown below:

$$\%\,\text{Crystallinity} = \left[\text{A (peak)} / \text{A (total)}\right] \times 100\,\%$$

2.4 | Experimental design and statistical analysis

The design used in this study was Completely Randomized Design (CRD) with experiments performed in triplicate. Data were analyzed by ANOVA and the Duncan's multiple range tests used to determine the significant differences ($p < .05$). Statistical analysis was carried out using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

3 | RESULTS AND DISCUSSION

3.1 | Effect of ultrasonication under different conditions on bio-calcium characteristics

3.1.1 | Particle size distribution

The mean particle sizes (D_{43}) of suspended bio-calcium powders were significantly influenced by ultrasonication treatments which resulted in a reduction of the values from 28.85 μm to 7–3 μm (Table 1). The amplitude and time of ultrasonication had profound effects on particle size of the bio-calcium powders ($p < .05$). The lowest ultrasonication amplitude (60%) reduced the particle size by 4 and 7 times for A60T15 and A60T30, respectively. Ultrasonication at higher amplitudes (70% and 80%) for different times (15 and 30 min) reduced the particle size of bio-calcium powder by about 10 times. However, no differences in particle size were observed among the sample subjected to 70% and 80% amplitudes, regardless of ultrasonication time ($p > .05$). The decrease in bio-calcium particle size was mainly due to the cavitation effect produced by ultrasound treatment. The cavitation generated shock waves through the liquid, which increased the local stress on suspended solids, thus inducing breakup and particle size reductions. This is consistent with a previous work, which reported cavitation also generated fragments of the particle as well as attrition on the surface, causing size reduction (Sumari et al., 2013). Particle size reduction by ultrasonication varies and is dependent on the material type and operating conditions. Bi et al. (2015) reported that avocado puree particle size was reduced from 52.31 to 13.44 μm by ultrasonication after 1 min. The fiber lengths of cellulose were reduced to 30–50 μm from the original value of 80–120 μm with 1 hr ultrasonication and became 20–30 μm with 5 hr treatment (Sumari et al., 2013). Similarly, the particle size of $LaCoO_3$ powders was decreased from 4.90 to 3.24, 2.89, and 1.74 μm after treating with ultrasonic bath for 2, 4, and 6 min, respectively (Sompech et al., 2012).

TABLE 1 Effect of different amplitudes and ultrasonication duration on the characteristics of bio-calcium suspension

Samples	Mean of particle size (μm)	Zeta potential (mV)	pH
Control	28.85 ± 3.47*a**	−13.41 ± 0.88a	7.13 ± 0.06a
A60T15	7.12 ± 0.86b	−13.15 ± 0.86a	6.95 ± 0.06b
A60T30	4.52 ± 0.19c	−12.22 ± 0.59a	6.72 ± 0.04c
A70T15	3.38 ± 0.41d	−12.37 ± 1.11a	6.84 ± 0.03c
A70T30	3.20 ± 0.59d	−12.87 ± 0.75a	6.75 ± 0.03c
A80T15	3.08 ± 0.36d	−12.91 ± 0.65a	6.78 ± 0.05c
A80T30	2.88 ± 0.33d	−12.59 ± 0.33a	6.78 ± 0.04c

Note: Bio-calcium powder was mixed with distilled water at a ratio of 1:4 (wt/vol) prior to ultrasonication. Control: Bio-calcium powder without ultrasonication treatment; A60T15: Bio-calcium powder treated with ultrasonication at 60% amplitude for 15 min; A60T30: Bio-calcium powder treated with ultrasonication at 60% amplitude for 30 min; A70T15: Bio-calcium powder treated with ultrasonication at 70% amplitude for 15 min; A70T30: Bio-calcium powder treated with ultrasonication at 70% amplitude for 30 min; A80T15: Bio-calcium powder treated with ultrasonication at 80% amplitude for 15 min; and A80T30: Bio-calcium powder treated with ultrasonication at 80% amplitude for 30 min.

*Values represent mean ± *SD* ($n = 3$).

**Different lowercase letters in the same column denote significant differences ($p < .05$).

Particle size distributions of ultrasonicated bio-calcium at different amplitudes and times are presented in Figure 1. Control bio-calcium powder (no ultrasonication) showed bi-modal particle size distribution with a broad curve, indicating a wide range of particle sizes. However, after the ultrasonication at 60% amplitude (A60T15 and A60T30), tri-modal particle size distribution was observed. Furthermore, at higher amplitudes (A70T15, A70T30, A80T15, and A80T30), bi-modal distributions particle sizes were noticed, in which smaller particle sizes were dominant. After ultrasonication at 60% amplitude, the distribution curve revealed that the proportion of particles sizes bigger than 28 μm was notably reduced, whereas the formation of the 3 μm particles was augmented considerably. The smaller particle size of ultrasonicated bio-calcium is consistent with a distribution curve that is narrower than observed for the control. Similarly, Yin et al. (2015) reported that micro

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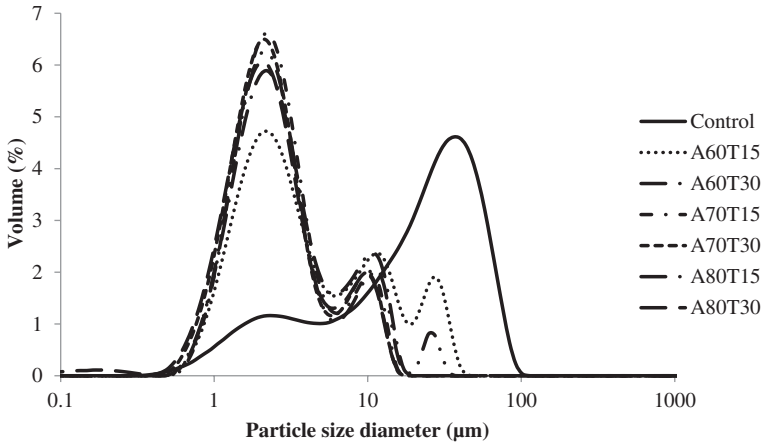

FIGURE 1 Particle size distribution of bio-calcium powder suspension at different amplitudes and duration of ultrasonication treatment

fishbone had a wider range distribution (1–100 μm) than the nano fishbone (10–100 nm) prepared by wet milling for 6 hr.

3.1.2 | Zeta potential

Zeta potential of ultrasonicated bio-calcium at different amplitudes and times measured at pH 7.2 is displayed in Table 1. Amplitudes and ultrasonication duration had no impact on zeta potential ($p > .05$). All samples showed a negative charge in the −12.22 mV to −13.41 mV range. Yin et al. (2015) reported that nano fishbone produced by wet media milling for 6 hr had a net negative charge of −11.4 mV at pH 7.0, due to the presence of hydroxyapatite (Hap) in the fishbone bio-calcium powder. The negative zeta potential at neutral pH was mainly attributed to the presence of phosphate groups in the Hap. The zeta potential of Hap varied with pH, which was negative at neutral and basic pHs, while positive at acidic pH (Doostmohammadi et al., 2012; Ahmed, Sheikh, & Zaki, 2015). The most important factor that affects the zeta potential is the pH of the medium (Lu & Gao, 2010). Zeta potential (ζ) can be negative, positive, or varied, depending on the measurement conditions. Zeta potential values can be used to predict the colloidal stability with typical values in the range of +100 to −100 mV (Shnoudeh et al., 2019). High stability of dispersion was usually observed at zeta potential with values >+25 mV or <−25 mV. Lower zeta potential is related with coagulation, flocculation, or aggregation, which are mediated by van der Waals inter-particle attractions (Shnoudeh et al., 2019). Control and ultrasonicated bio-calcium powders had zeta potential around −12 to −13 mV, which showed a low degree of stability. This coincided with the precipitation of bio-calcium suspension when stood for a long time.

3.1.3 | pH

Ultrasonication treatments at different amplitudes and duration gave different effects on the pH value of the bio-calcium powders ($p < .05$) as displayed in Table 1. The pH values of bio-calcium powder suspensions were reduced from 7.13 (control) to 6.72–6.95 for the ultrasonicated samples. However, no differences in pH were observed among ultrasonicated samples ($p > .05$), except A60T15 which had higher pH than the other samples with ultrasonication. ($p < .05$). The slight reduction in pH may have been as a result of proton release from protein degradation, especially collagen in the bio-calcium powder during the ultrasonication process. Small peptides containing acidic amino acids or free acidic amino acids might contribute to the decreased pH values of bio-calcium powder suspensions. It has been reported that a high ultrasonic intensity/amplitude (>60%) produced the growth and collapse of bubbles inside medium, resulting in the turbulence of system (Jambrak et al., 2007). Organic or inorganic substances can be decomposed by such turbulent ultrasonic waves, which affect pH values. For example, ultrasonic energy transition could react with the OH− or H+ ions, thus altering pH of the sample (Bhapkar et al., 2016). However, previous reports suggest that the effect of ultrasonication varies, depending upon the type and initial pH of the material. For example, decreases in pH occurred in cauliflower and brussels sprouts (Jambrak et al., 2007), sugarcane juice variants (Jasmi et al., 2019), baked eggs (Kang et al., 2016), and raw milk (Naeim, 2019) when subjected to ultrasonication. In contrast, increased pH values were found in ultrasonicated cabbage juice (Bhapkar et al., 2016) and fresh albumen (Sert et al., 2011).

3.2 | Characteristics of ultrasonicated bio-calcium as affected by different drying methods

Ultrasonication condition at 70% amplitude for 15 min (A70T15) was chosen as an efficient process to reduce the particle size of bio-calcium powder. Figure 2 shows bio-calcium powder suspension without ultrasonication (A) and with ultrasonication at 70% amplitude for 15 min after standing for 1 hr at room temperature. Bio-calcium powder suspension without ultrasonication was precipitated, whereas the ultrasonicated powder with smaller particle size remain suspended in water. Since the water was used as a medium for ultrasonication, drying was needed to improve long-term stability of the bio-calcium powder. The impacts of different drying

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FIGURE 2 (a) Fish bio-calcium powder suspension without ultrasonication and (b) with ultrasonication treatments at amplitude 70% for 15 min after standing for 1 hr at room temperature; (c) Control: Bio-calcium powder without ultrasonication; (d) BUF: Ultrasonicated and freeze-dried bio-calcium powder; and (e) BUR: Ultrasonicated and hot air (tray)-dried bio-calcium powder

TABLE 2 Effect of drying method on the proximate, mineral compositions, and calcium solubility of ultrasonicated bio-calcium powders

Parameters	Control	BUF	BUR
Moisture (%)	4.33 ± 0.03[+]a[+]	1.23 ± 0.1c	3.18 ± 0.06b
Protein[*] (%)	9.35 ± 0.23a	9.34 ± 0.07a	8.79 ± 0.09b
Fat[*] (%)	1.25 ± 0.08a	1.12 ± 0.04a	1.11 ± 0.03a
Ash[*] (%)	85.68 ± 0.27a	86.36 ± 0.23a	86.08 ± 0.11a
Hydroxyproline[*] (mg/g)	10.01 ± 0.65a	9.96 ± 0.47a	9.11 ± 0.94a
Calcium/Ca (%)[*]	33.72 ± 0.30a	33.43 ± 0.20a	33.7 ± 0.40a
Phosphorous/P (%)[*]	20.21 ± 0.20a	19.82 ± 0.10a	20.2 ± 0.20a
Mole ratio Ca/P	1.29	1.31	1.29
Ca solubility (%)	7.83 ± 0.01b	9.37 ± 0.01a	6.92 ± 0.01c

Note: Control: Bio-calcium powder without ultrasonication; BUF: Ultrasonicated and freeze-dried bio-calcium powder; and BUR: Ultrasonicated and hot air (tray)-dried bio-calcium powder.

[*]Dry weight basis.

[+]Values represent mean ± *SD* ($n = 3$).

[++]Different lowercase letters in the same row denote significant differences ($p < .05$).

methods were then investigated. Control bio-calcium, BUF, and BUR powder are shown in Figure 2c–e, respectively.

3.2.1 | Chemical composition

Proximate compositions, calcium, and phosphorus contents of ultrasonicated bio-calcium powders obtained from two different drying methods are given in Table 2. Different drying methods yielded slight effect on moisture content ($p < .05$) but no significant ($p > .05$) impact was observed on protein, lipid, ash, calcium, and phosphorus contents. After drying, moisture content of bio-calcium was drastically decreased and drying of suspensions with smaller particle size was more efficient due to the larger surface area, which improved heat transfer. Therefore, the moisture content of the control bio-calcium powder was higher than the ultrasonicated product (BUF and BUR). However, the moisture content of BUF was lower than that of BUR ($p < .05$). The moisture content of control bio-calcium,

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TABLE 3 Effect of drying method on the color and degree crystallinity of ultrasonicated bio-calcium powders

Color Parameters	Control	BUF	BUR
L^*	94.4 ± 0.2[+]a[+]	93.99 ± 0.09b	94.26 ± 0.14a
a^*	−0.16 ± 0.06a	−0.29 ± 0.04b	−0.23 ± 0.05ab
b^*	8.03 ± 0.57a	8.45 ± 0.22a	7.39 ± 0.17b
Whiteness index	90.2 ± 0.58ab	89.62 ± 0.18b	90.65 ± 0.21a
ΔE^*	7.72 ± 0.53a	8.09 ± 0.22a	7.05 ± 0.17b
Crystallinity (%)[#]	70.49	69.80	70.32

Note: Control: Bio-calcium powder without ultrasonication; BUF: Ultrasonicated and freeze-dried bio-calcium powder; and BUR: Ultrasonicated and hot air (tray)-dried bio-calcium powder.

[+]Values represent mean ± *SD* ($n = 3$).

[++]Different lowercase letters in the same row denote significant differences ($p < .05$).

[#]Crystallinity of raw bone: 52.60%.

BUF, and BUR powders were 4.33%, 1.23%, and 3.18%, respectively. During freeze-drying, the combination process including primary drying (sublimation), and secondary drying under vacuum took place, resulting in more efficient dehydration (Oyinloye & Yoon, 2020). Puranic et al. (2012) reported that freeze-drying could reduce the moisture content of garlic cloves to 98%, while hot air drying (65°C for 3 hr) could reduce the moisture content by only 13.3%. Other studies have also showed that freeze-drying generated lower moisture contents than hot air drying for several products such as garlic (Puranic et al., 2012), fish meat gel (Hu et al., 2013), fungus *Cordyceps militaris* (Chimsook, 2018), mango slices (Dereje & Abera, 2020), etc.

The control of bio-calcium powder showed similar protein content to BUF and BUR with no observed effect of drying. The results indicate that both ultrasonication and drying processes had no significant effect on protein content ($p > .05$) of the bio-calcium powder. However, previous works have showed that the protein content of fishbone bio-calcium powder varied, depending on pre-treatment and fish species. For example, fishbone bio-calcium powder prepared by autoclaving pre-treatment of Asian sea bass bone showed lower protein content than those from skipjack tuna (Benjakul, Mad-Ali, Senphan, et al., 2017), salmon (Idowu et al., 2020), tonggol, and yellowfin tuna bones (Benjakul, Mad-Ali, & Sookchoo, 2017) prepared by alkaline pretreatment.

Ultrasonication and drying method exhibited no influence on fat content of the bio-calcium powder. The fat content of control bio-calcium, BUF, and BUT powders were 1.25%, 1.12%, and 1.11%, respectively. The fat contents are higher than that of salmon bio-calcium powder (0.33%) prepared by alkaline pre-treatment, but lower than that of salmon bio-calcium powder (1.70%) prepared by non-alkaline pre-treatment (Idowu et al., 2020).

Ultrasonication and drying method had no impact on ash, calcium, and phosphorus contents of the samples. Ash, calcium, and phosphorus contents of all samples were 85.68%–86.36%, 33.43%–33.72%, and 19.82%–20.21%, respectively. The values are higher than those documented for bio-calcium powder from silver carp (Yin et al., 2016), skipjack tuna (Benjakul, Mad-Ali, Senphan, et al., 2017), and salmon (Idowu et al., 2020). Calcium and phosphorus molar ratios (Ca/P mole ratio) of the Asian sea bass bio-calcium powder (1.29–1.31) are lower than that 1.67 for pure Hap. The results indicate that the phosphorous content of bio-calcium powder is higher than that of pure Hap. The high content of phosphorus might be from phospholipid or phosphoprotein in the fishbone. Zhou et al. (2010) reported that herring fishbone contained non-collagenous phosphoproteins of the bone extracellular matrix. Herring by-product consisting of heads, fins, frames, and organs also contained phospholipids (Aidos, 2002). Additionally, the Asian sea bass backbone bio-calcium powder may contain other phosphate salts such as monosodium phosphate, monocalcium phosphate, and monoammonium phosphate, mainly from the diet (Morales et al., 2018), which could have led to the higher proportion of phosphorous.

3.2.2 | Solubility of bio-calcium in simulated gastrointestinal tract system

The solubility of bio-calcium powder in simulated gastrointestinal tract system is shown in Table 2. The drying method affected the solubility of ultrasonicated bio-calcium powder with BUF having a higher solubility than the BUR. The calcium solubility values of control, BUF, and BUR are 7.83%, 9.3%, and 6.92%, respectively. Calcium solubility was increased after ultrasonication treatment followed by freeze-drying. This might be due to the decrease in particle size as shown in Table 1 and increased porosity of the bio-calcium powder. Freeze-drying more likely resulted in a less compact structure with increased porosity (BUF), hence the solubility was slightly higher than the more compact hot air-dried product (BUR). This is consistent with previous reports that freeze-drying enhanced the porosity of Hap (Indrani et al., 2017; Román et al., 2011; Zhou et al., 2017). The solubility values obtained in this work are similar to those of bio-calcium powders derived from salmon, skipjack, yellowfin tuna frame (7.65-10.8%) which were also digested using simulated gastrointestinal tract fluids (Benjakul, Mad-Ali, Senphan, et al., 2017; Benjakul, Mad-Ali, & Sookchoo, 2017; Idowu et al., 2020).

3.2.3 | Color

The drying method had effect on the color parameters (L^*, a^*, b^*, whiteness index, and ΔE^*) of the samples (Table 3). BUF sample

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showed the lowest L^*-value, however there was no difference between control and BUR ($p > .05$). No difference in a^*-value was noticeable between BUF and BUR ($p > .05$). Yellowness was decreased in BUR as evident in the lower b^*-value. No difference in b^*-value between control and BUF was found ($p > .05$). Whiteness index of control denoted no difference with BUF and BUR ($p > .05$). However, a difference in whiteness was noticed between BUF and BUR ($p < .05$). The lowest ΔE^*-value was observed for BUR but no difference in ΔE^*-value between control and BUF was recorded ($p > .05$).

Freeze drying process made bio-calcium more porous, compared to rotary tray (hot air) drying method. Residual lipids of BUF could have been more exposed to oxygen than BUR, leading to higher b^*-value along with lower whiteness index, plausibly caused by the formation of lipid oxidation products. Oyinloye and Yoon (2020) suggested that during grinding of freeze-dried products, proper precautionary checks must be must be observed since bioactive compounds such as carbohydrates, proteins, lipids, and vitamins present in food are readily exposed with smaller particle size of products, which makes them susceptible to environment-induced alterations, especially in the presence of oxygen.

3.2.4 | X-ray diffraction (XRD)

XRD diffractograms of Asian sea bass raw backbone and bio-calcium powders are displayed in Figure 3. Raw backbone showed broader and merged peaks than all bio-calcium powder samples. Similar pattern and intensity of diffraction peaks were observed among control, BUF, and BUR powders. Hence, ultrasonication and different drying methods showed no impact on the XRD diffractogram of the bio-calcium powder. However, broad peaks in all bio-calcium powder samples were noticed as a result of the contribution from both elastic and inelastic scattering of hydroxyapatite nanocrystals (Londoño-Restrepo et al., 2019). Venkatesan et al. (2015) reported that raw salmon bone had the lowest intensity of XRD peaks compared to crushed, alkaline treatment, and calcined bone. Based on a reference data of the International Centre for Diffraction Data (ICDD) no. 00-064-0738, the XRD pattern of Asian sea bass raw backbone and bio-calcium powders are consistent with that of Hap (Veselinović et al., 2010).

The lowest degree of crystallinity (52.60%) was observed for the raw backbone as shown in Table 3. Similar degree of crystallinity was observed among control, BUF, and BUR powders. Effect of freeze-drying and hot air drying on degree crystallinity of several products varied, depending on the type of materials. Zang et al. (2014) reported that a lower degree of crystallinity of potato and maize starches was achieved by freeze-drying, compared to those from oven and ethanol drying. Freeze-dried gelatin hydrogel possessed amorphous chain conformation and porous structure, whereas the air dried hydrogels had non-porous and compact structures (Simoni et al., 2017). However, freeze-dried nata de coco (Pa'e et al., 2014) and nanocellulose (Peng et al., 2013) had slightly higher degree of crystallinity than oven-dried samples. Degree of crystallinity of all

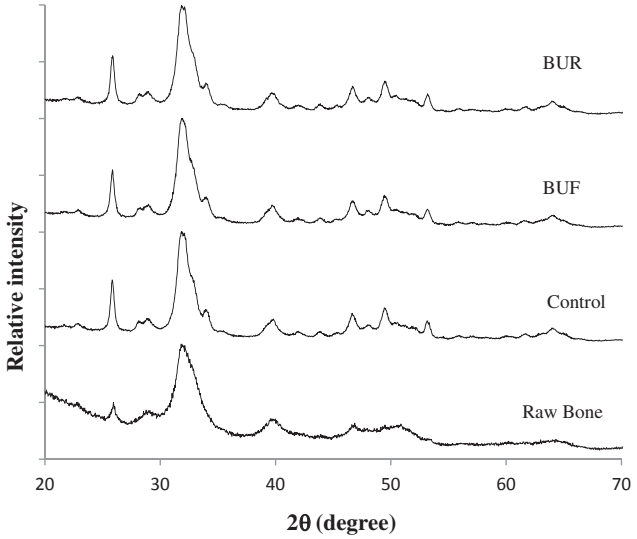

FIGURE 3 XRD diffractograms of Asian sea bass bio-calcium powder subjected to ultrasonication treatment followed by freeze-drying or hot air drying. Raw bone: Asian sea bass raw backbone used for bio-calcium powder production; Control: Bio-calcium powder without ultrasonication; BUF: Ultrasonicated and freeze-dried bio-calcium powder; and BUR: Ultrasonicated and hot air (tray)-dried bio-calcium powder

bio-calcium samples in this study was higher than that of bio-calcium from skipjack tuna (Benjakul, Mad-Ali, Senphan, et al., 2017), tonggol, and yellowfin tuna (Benjakul, Mad-Ali, & Sookchoo, 2017) prepared by alkaline pretreatment. Bio-calcium samples in this study were prepared by autoclaving pre-treatment of the fishbone. During heating under high pressure of the autoclave, alignment of crystal, especially Ca-hydroxyapatite, might be enhanced. Simultaneously, collagen could be leached out via degradation into water-soluble forms, which could have made the crystals become more compact. This was evidenced by increased crystallinity as well as the slightly sharper peak observed from the XRD diffraction patterns of bio-calcium samples than the raw backbone.

4 | CONCLUSIONS

Ultrasonication treatment of fishbone-derived bio-calcium powders at different amplitudes and duration had different effects on the particle size and pH but had no pronounced impact on zeta potential. Ultrasonication treatment at 70% amplitude for 15 min was selected since it led to the most effective reduction in particle size from 28.85 to 3.38 μm. The drying method had significant effect on moisture content and calcium solubility but had no impact on protein, fat, ash, calcium, and phosphorus contents. Calcium solubility increased after the ultrasonication treatment was followed with freeze-drying. Thus ultrasonication combined with freeze-drying is recommended for the production of bio-calcium powders with a less compact structure and potentially higher calcium bioavailability.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Investigation; Methodology; Writing-original draft: Wijayanti. *Validation; Writing-review & editing*: Sookchoo. *Validation; Writing-review & editing*: Prodpran. *Validation; Writing-review & editing*: Mohan. *Validation; Writing-review & editing*: Aluko. *Conceptualization; Funding acquisition; Project administration; Resources; Validation; Writing-review & editing*: Benjakul.

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