Optimization of Catfish (*Pangasius* sp*)* Bone Bio-calcium Production with Different Concentrations of Citric Acid and Stirring Time Using the Response Surface Method (RSM) Approach

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Abstract

Calcium is an important macro-mineral that is really needed by the body. Calcium from fish bones can be an alternative source of calcium for those who are allergic to milk lactose which is usually used as calcium source. The weakness of fish bone calcium is that it still has a fishy odor, which reduces the hedonic value of products fortified with fish bone calcium. This research aims to determine the optimization of concentration and stirring time in citric acid to produce bio-calcium from catfish (*Pangasius* sp*)* bones with the best calcium content and hedonic characteristics using Response Surface Methodology (RSM). The citric acid concentration tested was a minimum of 0.25% and a maximum of 1% with stirring times of 30, 60 and 90 min. Data processing was carried out with the help of Design Expert 11 software. The recommended model is the quadratic model. The recommended optimization results for the bio-calcium production solution are a citric acid concentration of 0.25% and a soaking time of 30 min. Verification was carried out according to the optimization solution and gave yield results of 88.9%, particle size of 51.07 µm, calcium content 24.05; with a color hedonic value of 4.115, aroma 4.059 and overall 4.090 out of a maximum value of 5. XRD pattern showed no difference between fishbone powder and bio-calcium with degree crystallinity 57 and 56.2%, respectively. FTIR spectra showed that both fishbone powder and bio-calcium powder contained hydroxyapatite, however citric acid slightly removed protein due to absence of amide 1 peaks in bio-calcium powder. Citric acid at 0.25% for 30 min helped to increase the yield and reduced some organic compound to obtain bio-calcium powder. The proper concentration of citric acid and stirring time yielded catfish bone bio-calcium with higher calcium content and hedonic value.

Keywords: catfish bone, citric acid, bio-calcium

Introduction

Calcium is very important for humans because it plays a role in forming and maintaining healthy bones and teeth (Fischer et al., 2018). Calcium is involved in several biological functions, for example muscle and blood vessel contraction, nerve transmission, intracellular signalling, hormonal secretion, and vasodilation (Beto, 2015). Therefore, calcium deficiency has a negative impact on bone and tooth health as well as human metabolism. Fish bones are high in calcium and phosphorus, 13.5-23.3% and 8.1-11.1%, respectively, but the content varies depending on the species. Bones also contain other minerals such as magnesium, iron, zinc, copper and others (Toppe et al., 2007).

Catfish (*Pangasius* sp*)* is one of the aquaculture products that continues to be developed. The production of catfish, which is around 124,412.55 tons in 2020, has encouraged catfish farmers not only to sell catfish in a fresh condition but also to process them to increase the value of the product. Processing frozen catfish fillets is an effort to increase fish consumption in the

community. Catfish fillet generates solid waste around 45% including heads, bones, skin and stomach contents is around 55% of the total weight of the fish (Nurilmala et al., 2018). Catfish waste, especially bones, is potentially used as a source of bio-calcium. The production of bio-calcium from fish bones were influenced by several factors, including pre-treatment using alkaline (Idowu et al., 2019; Benjakul et al., 2017a; 2017b), temperature and time of calcination (Chen et al., 2019) etc. Production of bio-calcium using alkali without pressure heating produced bio-calcium with the characteristics of gritty calcium powder so that its application in food is still limited. Previous research, namely the production of bio-calcium from the bones of barramundi (*Lates calcarifer*) using the pressure heating method, produces bio-calcium that is relatively applicable to various food products such as surimi, fish spread, apple juice and mayonnaise (Wijayanti et al., 2021a; Wijayanti et al., 2022a; 2022b; Wijayanti et al., 2023). However, the fishy odor of resulting bio-calcium was still detectable, improving method is still needed to decrease un-pleasant odor for its wider application into various food products.

Wijayanti et al. (2021b) extracted bio-calcium from the waste of Asian sea bass *(Lates calcarifer*) bones using the pressure heating method to reduce the use of chemicals NaOH and HCl. This bio-calcium was also applied to various food products. In vitro tests related to the solubility and bioavailability of biocalcium in the gastrointestinal system and Caco-2 cells have also been carried out (Wijayanti et al., 2022b). However, the volatile components in previous studies were still quite high (Wijayanti et al., 2023) so that the addition of calcium to foodstuffs was still limited. In addition, the use of hexane for fat removal and H_2O_2 in the bleaching process (Wijayanti et al., 2021b) were worried to have an impact on health even though the process has been continued to remove the chemical used. In this research, the fat removal and bleaching processes were eliminated. Citric acid was used to reduce fat and volatile compounds to decrease its fishy odor. Jo et al. (2005) showed that citric acid could remove trimethylamine (TMA) as representing fishy odor more than malic, lactic and acetic acids. Hence, citric acid was selected in this study. The concentration of citric acid had different effect on fishy odor of gelatin extraction (Sae-Leaw et al., 2016) and might have varying impact on fishy odor of fishbone calcium. However, study on effect of citric acid on fishbone calcium was limited. Moreover, time of stirring in the extraction process of calcium is important to obtain maximum yield and calcium content. Aprilia et al. (2020) reported that stirring time had significant effect on beta-tricalcium produced from *Anadara granosa* shells. This study aimed to optimize the production/ extraction of bio-calcium using the Response Surface Method (RSM) approach with success parameters being the yield and the highest calcium content with the lowest particle size. The RSM statistical method is used to ensure a quicker process and an analysis of the relationships between various factors for determining the optimal condition at the lowest total cost (Sriuttha et. al., 2024). The method for producing bio-calcium in this research is a modification of the method of Wijayanti et al. (2021b) by replacing the fat removal process with hexane and bleaching with H_2O_2 replaced with the use of citric acid. The treatments consisted of different concentrations of citric acid and length of stirring time. The study aimed to get the optimization condition of concentrations citric acid and time stirring to obtain bio-calcium powder from catfish (*Pangsius* sp*)* with high yield but less odor.

Materials and Methods

Material

The raw material used was the frozen of catfish (*Pangasius* sp*)* bone from fillet factory PT. Kurnia Mitra Makmur Purwakarta (KMMP), West Java, Indonesia. The frozen samples were sent from KMMP in the styrofoam box with ice ratio of 1:1 to Department Fisheries Product Technology, Faculty of Fisheries Marine Science within 4 h. Before used, sample was kept in freezer -20°C maximum 1 month.

Preparation Fishbone Coarse Powder

Fish bones were boiled at 100°C for 60 min to remove adherent fish meat (boiled bone/BB). Cleaned bone were autoclaved at 121C with pressure 2 atm for 90 min. Furthermore, sample was dried at 60 °C for 48 h and grinded to attain coarse powder.

Bio-Calcium Production

The fishbone coarse powder was produced for bio-calcium. The lipid of coarse powder was removed by soaking the samples in citric acid (LabScan, RCI Labscan Ltd., Bangkok, Thailand) at different concentrations (0.25, 0.625 and 1%) (w/v) and stirred for different times (30, 60 and 60 min) at 25°C (room temperature, RT). The minimum and maximum of concentrations of citric acids and stirring time were selected due to our previous study that the use of more than 1% citric acid made the resulted powder very hygroscopic, and more than 60 minutes of stirring generated a high yellow color of the resulted powder. Further, the solvent was drained, and the sample was neutralized using distillation water until its pH reached around 6.5-7. After being neutralized, the samples were

dried in the hot air oven at 60 C for 48 h. The dried powder (50 g) was placed in a grinding jar and ball milled using 4 grinding balls (diameter of 20 mm) at a speed of 200 rpm for 2 hours. The obtained powders were sieved using a sieving machine to collect particles of homogeneous size.

Formulation Preparation and RSM Design

RSM analysis was performed using software of Design Expert 11. The RSM experimental design used in the optimization stage was the Box Behnken Design. There are 2 independent variables used in this study including citric acid concentrations and stirring times. The range for each factor is determined by the rule that the distance between the midpoint (0) and the lower limit (-1) must have the same difference as the distance between the midpoint (0) and the upper limit (+1). The desired response is the yield, particle size, calcium content and hedonic value including color, odor and overall. The range of values on independent variables expressed in Table 1. Suggested research design was displayed in Table 2.

RSM Analysis

Response surface method (RSM) analysis was performed by determining the statistical model first. The model was developed using the Sequential Model of Sum Square, Lack of Fit Tests, and Statistics Summary. Analysis of Variance (ANOVA) was used to examine the effect of each component in the response. Diagnostic revealed the residual in the obtained model. The model graphs displayed in the form of contours and reaction curves depended on the modelling results.

Optimization Stage

Using the Design Expert 11 application, the yield, calcium content, particle size, and hedonic value (color, odor, and overall) would be optimized. For each factor, the program would propose an optimization solution. The best formula had the highest desirability value, which was near to 1 (Adamu et al., 2022). The optimization solution was validated to ensure that the results achieved were consistent with the program predictions. The yield, calcium content and particle

Table 1. Range of values on independent variables

Component	Independent Variable	Minimum	Maximum
A	Citric acid concentrations	0.25%	1%
B	Stirring time	30 min	90 min

Table 2. Research Design using Response Surface Method (RSM)

Run	Factor 1	Factor 2
	A-Citric Acid concentrations (%)	B-Stirring times (min)
R1	0.625	90
R2	1	60
R3	1	90
R4	0.625	60
R5	0.25	90
R6	0.625	60
R7	0.625	60
R8	0.625	60
R9	0.625	60
R10	0.625	30
R11	0.25	30
R12	0.25	60
R13	1	30

size were used for validation of the consistency of the program prediction. Fishbone powder without extraction using citric acid was analyzed for comparison.

Procedure Analyzes

Yield

Yield value of fishbone bio-calcium powder was determined by calculating with the written formula below (Kusumawati et al., 2022) :

$$\text{Yield value } (\%) = \frac{\text{Dried extracted bio-calcium (g)}}{\text{Fishbone powder (g)}} \times 100\%$$

Particle Size

Particle size was determined by Laser Particle Size Analyzer (LPSA) (Labtron, LLPA-C10) completed with automatic unit integrated with wet and dry dispersion. Samples were initially dispersed using water and then being read with the size measuring range of 0.01 µm until 2000 µm (Benjakul et al., 2017b).

Calcium

Calcium is determined according to the method as described by Idrus et al. (2022). Sample is mixed with DI water and homogenized to a concentration of 5 g/ 100 g. The calcium concentration in the dilution is measured with atomic absorption spectroscopy (AA-6300c, Shimadzu, Kyoto, Japan).

Hedonic Value

Hedonic testing was carried out to find out preference of the panellists on fishbone bio-calcium. Parameters in this hedonic test include color, odor and overall texture The scores used to assess a product included 1 (dislike very much), 2 (dislike), 3 (neutral), 4 (like), 5 (very like) (Waimaleongora ek & Prinyawiwatkul, 2021) Hedonic testing was carried out by thirty un-trained panellists by giving scores to product quality attributes. The hedonic evaluation took place in a well-lit, quiet, and odor-free space. The hedonic test was carried out by presenting samples in small containers for each panellist. Each sample was codified with three randomized diûerent numbers. The panellists were asked to give the score for color, odor and texture of catfish bone bio-calcium in the available forms.

Digital Image

A digital image of fishbone powder (control) and all bio-calcium samples were taken by mobile phone camera of the iPhone 11.

Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

The chemical structure of fish bone powder was analyzed following a previous method as described by Jiang et al. (2020), using a Fourier transform infrared spectroscopy (FT-IR) spectrometer (Nexus-470, Nicoletnexus, Japan). Samples are prepared as translucent KBr pellet and scanned against a blank KBr pellet background at the wavenumbers ranging from 4000 to 400 cm^{-1}.

X-ray Diffraction Analysis

The phase composition of samples is determined by X-ray diffraction (XRD) as described by Benjakul et al (2017b) using an X-ray Diffractometer (X' Pert MPD, PHILIPS, Eindhoven, the Netherlands), with Cu K-á radiation. The powder samples are scanned at 2è angle ranging from 20° to 80° with the scan speed of 3°/min and the step size of 0.05°at 40 kV and 30 mA. Phase identifications is performed by using a peak profile matching to the standard powder diffraction data file (JCPDF). Percentage of crystallinity was calculated by the following equation:

$$\% \text{ Crystallinity} = \left[\frac{\text{A peak}}{\text{A total}}\right] \text{x } 100$$

Where

A peak = Total area under all crystalline peaks

A total = Total area of all crystalline and amorphous peaks

Results and Discussions

The value of responses parameter as affected by different concentrations of citric acid and different times of stirring are displayed in Table 3.

Yield

The different concentrations and stirring times had different effect on yield of bio-calcium. ANOVA for linear model on yield (Table 4) showed that only concentrations that had significant effect on yield of bio-calcium powder ($p<0.05$), nevertheless the time of stirring had no significant effect ($p>0.05$). The resulted model for yield response was significant. The highest yield was attained for R2 when bio-calcium extracted using citric acid concentration of 0.25%. The lowest yield was achieved by R10 samples when bio-calcium extracted using 1% citric acid for 90 min. This yield responses showed that the higher concentration of citric acid the lower yield was attained, and the final equation prediction obtained was Yield =

Table 3. Yield, calcium content, particle size, hedonic value of catfish bio-calcium powder as responses of varying citric acid concentrations and stirring times

Sample code	Citric acid concentration (%)	Stirring time (min)	Yield (%)	Calcium content (g/100 g)	Particle size (µm)	Hedonic value		
						Odor	Color	Overall
R1	1	30	80	23.046	58.663	3.23	3.30	3.33
R2	0.25	90	89.9	24.958	61.239	3.20	3.77	3.37
R3	1	60	82.8	24.317	57.069	3.63	3.97	3.77
R4	0.625	90	82.6	19.265	53.834	3.47	3.57	3.67
R5	0.625	60	82.5	22.700	53.739	3.67	3.70	3.70
R6	0.625	60	81	20.053	59.261	3.73	3.87	3.83
R7	0.625	60	86.8	21.439	58.463	3.70	3.93	3.93
R8	0.25	60	88.5	18.950	56.75	3.63	3.73	3.80
R9	0.625	60	82.8	22.006	61.397	3.83	3.87	3.87
R10	1	90	74.2	23.761	58.086	3.90	4.10	3.97
R11	0.625	30	78.6	23.561	54.212	3.90	3.83	4.00
R12	0.625	60	81.4	21.849	58.129	3.87	3.73	3.93
R13	0.25	30	88.3	23.803	57.899	3.87	4.13	4.03

Table 4. ANOVA of yield response

Source	Sum of squares	df	Mean square	f-value	p-value	Statement
Model	**147.02**	**2**	**73.51**	**8.53**	**0.0069**	**Significant**
A-Citric acid concentration	147.02	1	147.02	17.05	0.0020	
B-Extraction time	0.0067	1	0.0067	0.0008	0.9784	
Residual	86.21	10	8.62			
Lack of fit	**64.97**	**6**	**10.83**	**2.04**	**0.2556**	**Not Significant**
Pure error	21.24	4	5.31			
Cor total	233.23	12				

83.03- 4.95 A -0.0333 B. The response surface plot of yield was drawn for investigation. Sriuttha et al. (2024) reported that calcium extraction from catfish bone using citric acid (0.1-0.25 M) assisted by microwave power 300 W resulted a higher yield at optimum concentration of 0.1 N using RSM with calcium content as a response. The yield of bio-calcium in this study was 74.2-89.9%. The lower yields (20-50%) were observed for nano-calcium produced by different fish species (tuna, kingfish mackerel, snapper and grouper) which were processed using chemicals (HCl and NaOH) and combined with three times autoclaving (Kusumawati et al., 2022). Figure1 shows the effect of citric acid concentrations and stirring time on yield of resulted bio-calcium. The decrease of yield with the rise of citric acid concentration might be due to the increase soluble compound of bio-calcium as affected by the higher concentrations of citric acid. Several mineral compounds such as calcium, phosphorous, etc. might be solubilized and then remove when citric acid was drained, hence the yield decreased. Several studies showed that citric acid could solubilize some minerals complect including phosphate (Porto et al., 2018; Lazo et al., 2017), calcium phosphate (hydroxyapatite) (Samavini et al., 2018; Barrow et al., 2018; Aenglong et al., 2022); Iron oxide (Olvera Venegas et al., 2014), etc.

Calcium Content

Varying concentrations of citric acid and stirring time had no different effect on calcium content of bio-calcium ($p>0.05$). The model was not significant, while the lack of fit was significant (data was not shown).

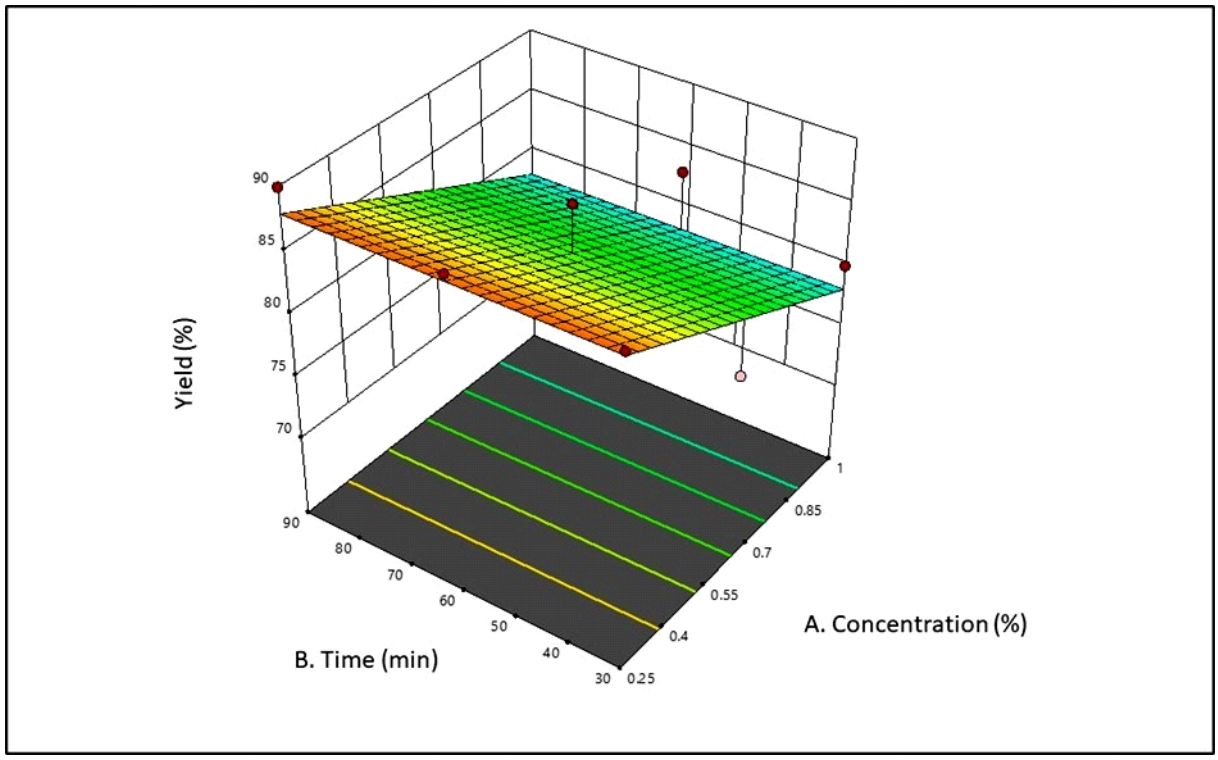

Figure 1. 3D diagrams of signiûcant interactions, the effect of citric acid concentration and stirring time on yield of bio-calcium

The lack of fit should be not significant or >0.05. The model can be appropriate if the p-value in the lack of fit test is more than 0.05 (Deng & Chen, 2019). Hence, the model was not appropriate for calcium content response. No difference on calcium content among running samples (R1-R13). It showed that the concentration of citric acids up to 1% and times of extraction were not significant to solubilize calcium of catfish bone powder. Sriuttha et al. (2024) reported that using RSM, the lowest concentration of citric acid (0.1 M) was also resulted the optimum calcium content compared to the higher concentrations (0.15, 0.20 and 0.25 M). Wijayanti et al. (2022b) reported that the highest calcium dissolution of Asian sea bass bone powder was observed when it was solubilized in 9% citric acid. All running samples in current study had calcium content around 18.95-24.95%. Calcium content of fishbone powder varied (15.47-30.67%) depending on species (Kusumawati *et al.*, 2022), chemical treatment method (Savlak *et al.*, 2020; Wijayanti et al., 2021b), temperature (Hammood et al., 2019; Zairin *et al.*, 2018), etc.

Particle Size

Different concentrations of citric acid and stirring time gave no different effect on particle size of bio-calcium ($p<0.05$). The model was not significant, while the lack of fit was significant (Table of ANOVA was not shown). The lack of fit should be not significant or >0.05. Deng and Chen (2019) reported that if the p value in the lack of fit test is more than 0.05, the model can be said to be appropriate. Hence, the model was not appropriate for calcium content response. No difference on particle size among running samples (R1-R13). The particle size of bio-calcium in this result was bigger (53.79-61.239 µm) than bio-calcium produced from Asian seabass (16.71 µm) (Wijayanti et al., 2021b) due to different method of milling process. Akpan et al. (2021) reported a higher particle size (100 mm) of hydroxyapatite from catfish bone which was produced by calcination at 900°C for 2 h 22 min. The lower particle size (0.73-1.03 µm) was observed for catfish bone powder extracted using lime solution which was assisted with ultrasonication process (Jasmadi et al., 2022).

Hedonic Value

Odor

Table 5 show the ANOVA hedonic of odor. The result showed that the combination of citric acid concentrations and stirring time give different effect on hedonic value of odor ($p<0.05$). However, each factor including citric acid concentrations and extraction time had not significant impact on odor, respectively ($p>0.05$). The model's accurate prediction of the

Table 5. ANOVA of hedonic of odor response

Source	Sum of squares	df	Mean square	F-value	p-value	Statement
Model	**0.4782**	**3**	**0.1594**	**7.37**	**0.0085**	**Significant**
A-Citric acid concentration	0.0007	1	0.0007	0.0309	0.8643	
B-Extraction time	0.0308	1	0.0308	1.42	0.2631	
AB	0.4467	1	0.4467	20.65	0.0014	
Residual	0.1946	9	0.0216			
Lack of fit	**0.1649**	**5**	**0.0330**	**4.43**	**0.0872**	**Not significant**
Pure error	0.0298	4	0.0074			
Cor total	0.6728	12				

hedonic odor was suggested by the low probability ($p<0.05$) and F value (7.37). The model was accurate for the experiment, as indicated by the un-significant lack of fit ($p>0.05$) which further demonstrated that it is not considerably relative to the pure error (Table 5). The final prediction equation attained was hedonic value of odor = 3.66+0.0106A-0.0717B. The highest color value was obtained for R13 with citric acid concentration of 0.025% and stirring time 30 min.

The compounds associated with off-flavors and off-odors are mainly produced by lipid oxidation, protein degradation, and microbial action (Zhang et al., 2023). Citric acid could increase the hedonic value of odor might be due to its capability to reduce the lipid content, hence the secondary lipid oxidation products which is one of the main causes of un-pleasant odor was inhibited. Sae-Leaw et al. (2016) reported that citric acid pre-treatment of gelatin production yielded less fishy odor than those using acetic acid due its capacity to decrease TBARS value as secondary lipid oxidation products. Citric acid was reported to remove muddy off-flavors in channel catfish (*Ictalurus punctatus*) that its suggested reduction might be the result of leaching of fat and protein and/or chemical dehydration (DeWitt et al., 2007).

Color

Different concentrations of citric acid and stirring time gave no different effect on color of bio-calcium ($p<0.05$). Using ANOVA, the model was significant, and lack of fit was not significant (Table 6). The final prediction equation attained was hedonic value of color = 3.81-0.0450A+0.0283B+0.2908AB. The highest odor value was attained for R10 with citric acid concentration of 1% and stirring time 90 min. However, the similar odor value was achieved by R11 which was extracted using 0.625% of citric acid stirred 30 min. Figure 2 showed that control bio-calcium (without citric acid treatment) had darker color than those treated with citric acid at different concentration (R1-R13). Citric acid assisted the color of bio-calcium become lighter. The color of bio-calcium extracted using 0.25% citric acid for 30 min (R13) showed the highest hedonic color value. The lower hedonic color value was observed for bio-calcium at higher concentration of citric acid.

The lighter color of bio-calcium treated with citric acid might be due to its lower oxidation product since citric acid reduce the lipid and protein content which could be potentially oxidized. Alimi and Workneh (2018) reported that lighter color was observed for citric acid modified starch due to the reduce of lipid and protein content as impurities.

Overall

Table 7 showed that different concentrations of citric acid and different times of stirring gave different effect on overall hedonic value. ANOVA of quadratic model showed significant effect and lack of fit was not significant. The prediction equation obtained in this quadratic model was overall hedonic value = 3.87-0.0233 A-0.0600 B+0.3250 AB-0.1126 A-0.0626 B. The model showed that the higher concentration of citric acid and time extraction would reduce the preference of panellists on bio-calcium powder. The higher citric acid generated the less appearance and color with no difference on odor. Thus, the lowest concentration of citric acid (0.25%) resulted the highest overall hedonic value. The use citric acid at some level could increase hedonic value of food products. Elibaid (2019) reported that citric acid treatment on beef burger could be antibacterial agent and increased hedonic values of beef burger. Citric acid at proper level in the beverage formulation which had the highest antioxidant capacities was the preferred product based on the hedonic evaluation (Oboh & Imafidon, 2018).

Table 6. ANOVA of hedonic of color response

Source	Sum of Squares	Df	Mean Square	F-value	p-value
Model	0.3553	3	0.1184	4.74	0.0300 significant
A-Citric acid concentration	0.0121	1	0.0121	0.4865	0.5031
B-Extraction time	0.0048	1	0.0048	0.1929	0.6709
AB	0.3383	1	0.3383	13.55	0.0051
Residual	0.2248	9	0.0250		
Lack of Fit	0.1857	5	0.0371	3.80	0.1102 not significant
Pure Error	0.0391	4	0.0098		
Cor Total	0.5801	12			

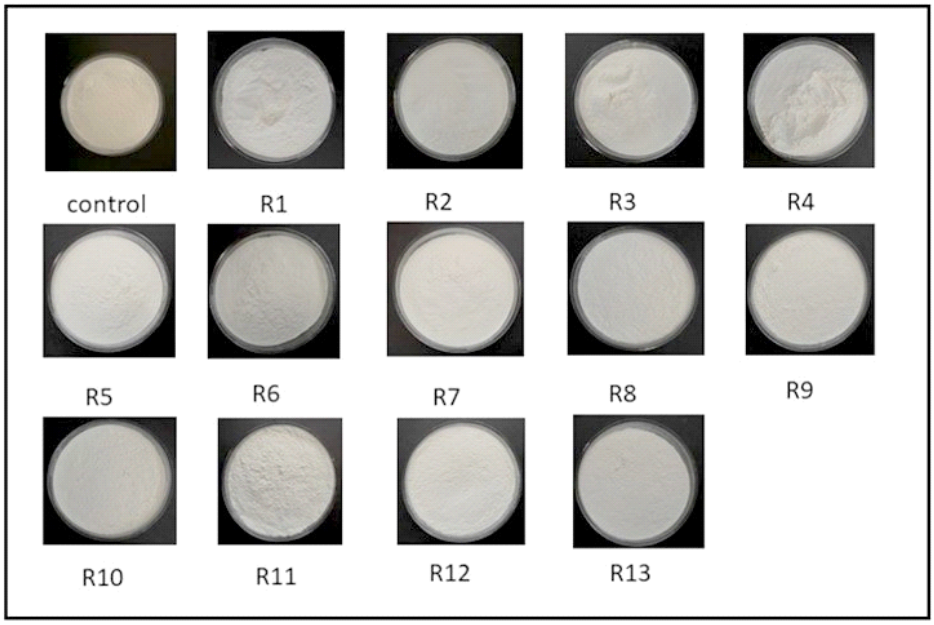

Figure 2. Bio-calcium extracted using different citric acid concentration and stirred for different times.

Table 7. ANOVA of overall hedonic value

Source	Sum of squares	df	Mean square	F-value	p-value	Statement
Model	**0.5184**	**5**	**0.1037**	**9.48**	**0.0051**	**Significant**
Citric acid concentration	0.0033	1	0.0033	0.2988	0.6016	
Extraction time	0.0216	1	0.0216	1.98	0.2027	
AB	0.4225	1	0.4225	38.64	0.0004	
A^2	0.0350	1	0.0350	3.21	0.1165	
B^2	0.0108	1	0.0108	0.9913	0.3526	
Residual	0.0765	7	0.0109			
Lack of fit	**0.0396**	**3**	**0.0132**	**1.43**	**0.3576**	**Not significant**
Pure error	0.0369	4	0.0092			
Cor total	0.5949	12				

Optimization

The citric acid concentrations variable was optimized in the range of 0.25% - 1% with an importance level of 3 (+++). The time of stirring variable was optimized in the range of 30-90 min with an importance level of 3 (+++). The yield, calcium content, particle size, hedonic value of odor, color and overall were also optimized with an importance level of 3 (+++). The constraint of optimization process is shown in Table 8. Based on the optimization process, Design expert 11 provided 1 optimum solution formula. The optimization solution of protein can be seen on Table 9.

The optimization solution results can be seen on Table 9. Based on Table 9 at concentration of citric acid of 0.25% and stirred for 30 min with a predicted yield, calcium content, particle size, hedonic of odor, color and overall were 88.014%, 57.595 mm, 23.492%, 4.115, 4.059 and 4.099, respectively. The value of desirability is 0.758 which was the closest with 1. The desirability value is closer to 1 means the ability of the program to produce the desired product will be more perfect (Adamu et al., 2022).

Validation and Verification

Validation process of bio-calcium was done for citric acid at 25% stirred for 30 min on yield, particle size and calcium content in comparison with the control (fishbone powder without citric acid treatments). The data validation is displayed in Table 10.

The yield of bio-calcium slightly reduced (in comparison to control) after extracted using citric acid for 30 min. It might be due to several compound which was soluble in the citric acid was removed when the citric acid solution was drained. Furthermore, neutralization process using distilled water was also decrease the yield of bio-calcium to remove several water-soluble compounds when the water removed. Calcium content of bio-calcium was higher than its fishbone powder. The calcium was concentrated when citric acid was used, since non-calcium compounds which were soluble in citric acid and water removed at drain process. Particle size of bio-calcium was lower than fishbone powder due to extraction process using citric acid slightly reduce the particle size. Particle size distribution curve of fishbone powder and bio-calcium treated with citric acid is shown in Figure 1.

Table 8. Constraint of the independent and dependent variable

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A-Citric acid concentration	Is in range	0.25	1	1	1	3
B-Extraction time	Is in range	30	90	1	1	3
Yield	Maximize	74.2	89.9	1	1	3
Particle size	Minimize	53.739	61.397	1	1	3
Calcium content	Maximize	18.95	24.958	1	1	3
Hedonic (Odor)	Maximize	3.33	4.13333	1	1	3
Hedonic (Color)	Maximize	3.33	4.13333	1	1	3
Hedonic (Overall)	Maximize	3.33	4.03333	1	1	3

Table 9. Optimization

No.	Citric acid concentration	Extraction time	Yield	Particle size	Calcium content	Hedonic (color)	Hedonic (smell)	Hedonic (overall)	Desirability	
1	0.250	30.000	88.014	57.595	23.492	4.115	4.059	4.099	0.758	**Selected**
2	0.250	35.028	88.009	57.595	23.492	4.071	3.991	4.053	0.749	
3	0.949	90.000	78.722	57.595	23.492	4.049	3.890	3.919	0.585	
4	0.954	90.000	78.650	57.595	23.492	4.052	3.895	3.921	0.585	
5	0.940	90.000	78.845	57.595	23.492	4.043	3.881	3.917	0.585	
6	0.961	90.000	78.562	57.595	23.492	4.057	3.901	3.923	0.585	
7	0.929	90.000	78.986	57.595	23.492	4.036	3.872	3.913	0.585	
8	0.889	90.000	79.512	57.595	23.492	4.009	3.835	3.899	0.583	

Table 10. Yield, calcium content and particle size of fishbone powder (control) and bio-calcium produced using citric acid at 25% stirred for 30 min

Parameters	Control (fishbone powder)	Bio-calcium extracted using citric acid 2.5% stirred for 30 min
Yield (%)	100	88.9±0.54
Calcium content (g/100g)	20±0.23	24±0.05
Particle size (μm)	65±0.54	51±0.17

Monomodal (one peak) pattern was observed in all samples and a slight difference in the size of the curves was observed. Fishbone powder (control) showed the narrow shape, while bio-calcium with 0.25% citric acid showed the sharper peak which show the lower particle size. Monomodal pattern of distribution particle size was also observed for fishbone bio-calcium powder other studies (Benjakul et al., 2017a; Wijayanti et al., 2021b; Idowu et al., 2020). Citric acid had been known as organic solvent to solubilize several compounds and remove them from the system. Citric acid has wider application including gelatine extraction to remove lipid (Sae-Leaw et al., 2016); demineralization process during chitin extraction (Pohling et al., 2022); solubility of hydroxyapatite nanoparticles (Samavini et al., 2018); etc.

X-Ray Diffraction (XRD)

XRD diffractograms of fishbone powder and bio-calcium from catfish bone are displayed in Figure 4 A and B, respectively. Similar pattern and intensity of diffraction peaks were observed among control and fishbone bio-calcium. Fishbone powder without citric acid treatment (control) (Figure 4A) and threated with 2.5% citric acid for 30 min showed no impact on the XRD diffractogram of bio-calcium. However, broad peaks in control and bio-calcium samples were observed as a result of the contribution from both elastic and inelastic scattering of hydroxyapatite nanocrystals (Londoo-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 (ICCD) no. 00-064-0738, XRD pattern of control and bio-calcium powder were consistent with that of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (Veselinoviae et al., 2010). Degree of crystallinity of control and bio-calcium powder were 57% and 56.2%, respectively. It showed the low degree of crystallinity was noticed in control and resulted bio-calcium powder. The degree crystallinity of catfish bio-calcium powder in this study was lower than those bio-calcium produced from Asian sea bass bio-calcium reported by Wijayanti et al (2021b). During heating under high pressure, alignment

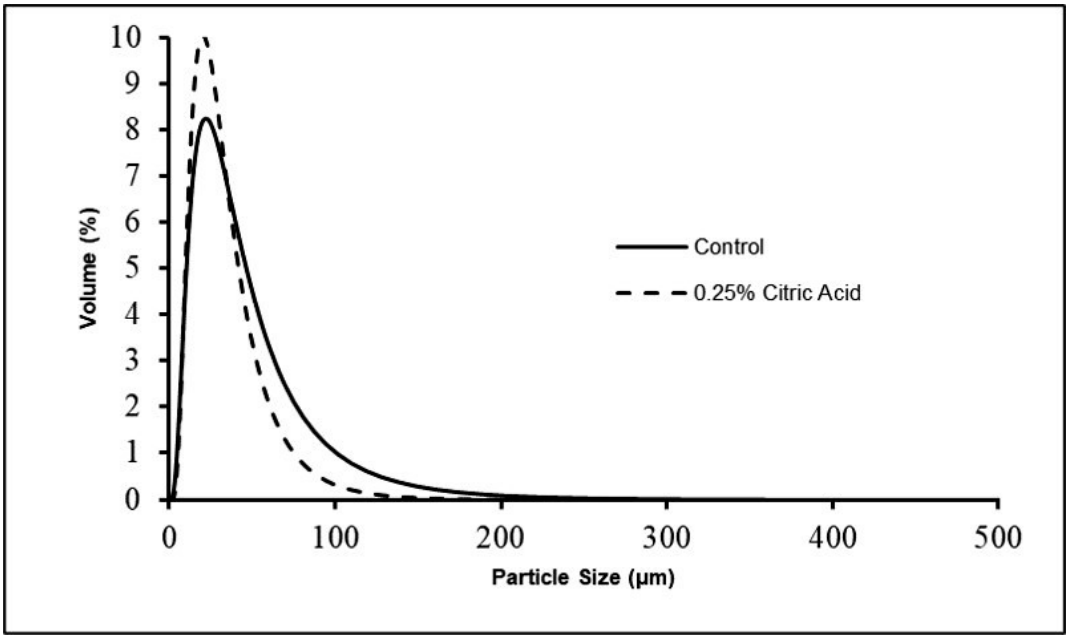

Figure 3. Particle size distribution of fishbone powder and bio-calcium treated with 25% citric acid

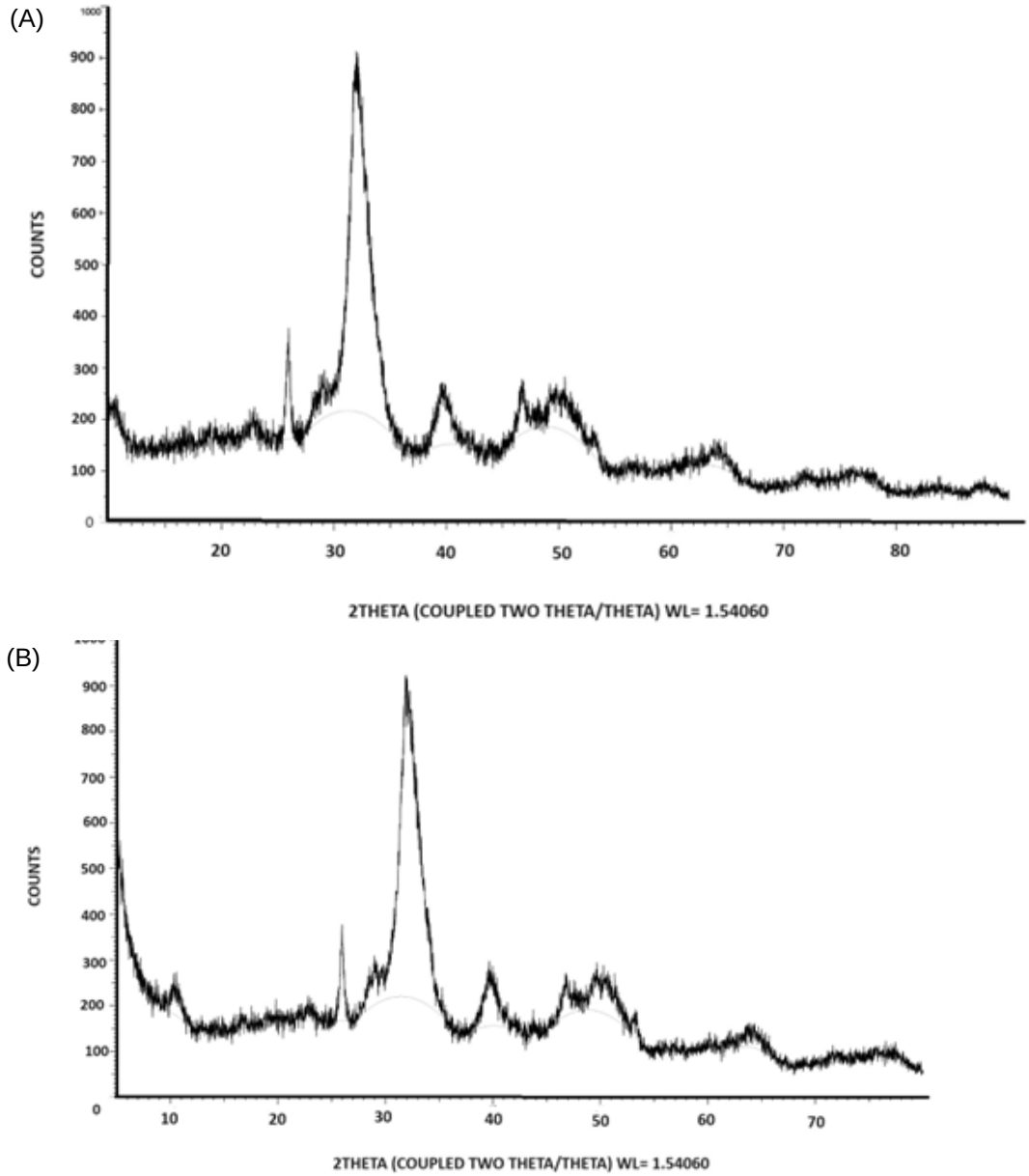

Figure 4. XRD Diffractogram of catfish fishbone powder (A) and bio-calcium powder treated with 0.25% citric acid (B)

of crystal, especially Ca-hydroxyapatite, might be enhanced. Simultaneously, collagen could be leached out via solubilization into water soluble form. As a result, the crystals became more compact. This was evidenced by increased crystallinity as well as the slightly sharper peak observed from the XRD diffraction patterns.

FTIR

FTIR spectra of bio-calcium from fishbone powder (control) and bio-calcium powder are displayed in Figure 5 A and B, respectively. Both samples had similar spectra but showed slightly the difference in amplitude. The band 3284.33 cm^{-1} and 3284.3 cm^{-1} of control (A) and bio-calcium powder (B), respectively, showed O-H stretch (hydroxyl group). Protein was also present as appeared by peaks 1637 cm^{-1} (amide I) and 1541 cm^{-1} (amide II) in control sample but only Amide II was observed in bio-calcium powder. It showed that citric acid had an impact on the decrease of protein content as consequence the amide I was not detected in citric acid treated bio-calcium. Benjakul et al (2017a) documented that the absorption peak at 1633 cm^{-1} and 1550 cm^{-1} in bio-calcium were amide I and amide II, respectively which are typical for the coiled structure of collagen. Kristoffersen et al. (2019) reported that

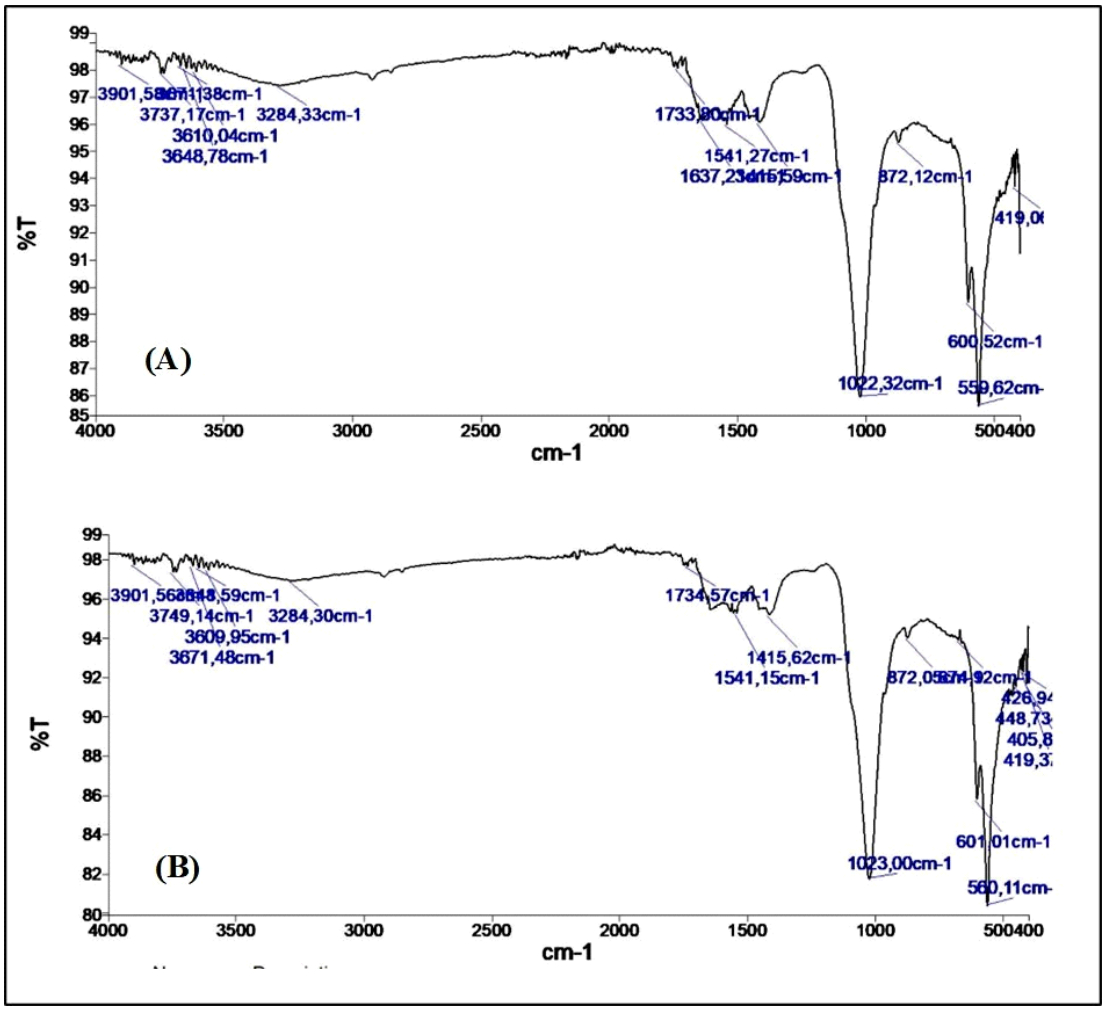

Figure 5. FTIR of catfish fishbone powder (A) and bio-calcium treated with 0.25% citric acid

the amide I (1650 cm^{-1}) and amide II (1550 cm^{-1}) bands were observed in protein hydrolysates. Sukumaran (2017) explained that the absorptions associated with C=O stretching is denoted as Amide I, whereas those associated with N—H bending is related with Amide II. The amplitudes of amide I and amide II bands of control were higher than those of citric acid treated bio-calcium powder. Both samples contained hydroxyapatite ($Ca_5(PO_4)_3(OH)$) as elucidated by FTIR spectra. A large peak was found at 1022 and 1023 cm^{-1}, representing the stretching mode of PO_4 vibration. The bands at 559 and 560 cm^{-1}, confirming the n4 symmetric P–O stretching vibration of a PO_4 group, were also noticed. Furthermore, the band at 3671 and 3648 cm^{-1} corresponded to the O-H stretch of hydroxyapatite (Pal et al., 2017). Several studies observed these FTIR spectra in raw fish bone (Pal et al 2017), fish bone bio-calcium (Benjakul et al., 2017a) and fish bones hydroxyapatite (Venkatesan et al., 2015; Boutinguiza et al., 2017; Pal et al., 2017).

Conclusions

Using the Box Behnken Design optimization, this current study found that 0.25% citric acid concentration and 30 min soaking time are suggested for optimization process of catfish bio-calcium based on yield, calcium content, particle size and hedonic value. Fishbone powder and citric acid treated bio-calcium, showed no difference on the XRD pattern. FTIR spectra revealed that hydroxyapatite was present in both catfish bone powder and citric acid treated bio-calcium powder. However, protein was somewhat eliminated by citric acid in the bio-calcium powder due to the absence of amide 1 peaks. Bio-calcium treated with 25% citric acid and stirred for 30 min increased the yield and decreased certain organic compound. The proper concentration of citric acid and stirring time yielded catfish bone bio-calcium with higher calcium content and hedonic value.

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Supplementary Materials

Supplementary materials is not available for this article

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