

RESEARCH ARTICLE

## Characterization of $\beta$ -tricalcium phosphate derived from green mussel shells (Molarity variation)

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Submitted: 12<sup>nd</sup> October 2023; Revised: 28<sup>th</sup> November 2023; Accepted: 4<sup>th</sup> December 2023

### ABSTRACT

$\beta$ -Tricalcium phosphate ( $\beta$ -TCP) is a widely used bioceramic material. In dentistry, it is commonly used as bone graft material.  $\beta$ -TCP is osteoconductive, bioresorbable, bioactive, and has biocompatibility properties. This study aims to evaluate the optimum molarity of CaO and H<sub>3</sub>PO<sub>4</sub> to synthesize  $\beta$ -TCP from a natural source (*Perma viridis linn*). This is laboratory experimental research conducted by reacting calcium compounds from green mussel shells and phosphoric acid using the dissolution precipitation method with variations in molarity ratio. X-ray diffraction (XRD), scanning electron microscope (SEM), and fourier transform infrared (FTIR) were used to identify the characteristics of  $\beta$ -TCP synthesized from green mussel shells. The XRD chart pattern showed the formation of peaks identical to the  $\beta$ -TCP (Sigma-Aldrich). However, formation of whitlockite phase was also seen in the results. FTIR results showed that phosphate, hydroxyl, and carbonyl groups were shown on the graph and could be identified as  $\beta$ -TCP. SEM characterization showed that the sample consisted of small particles irregularly shaped to form like aggregates.  $\beta$ -TCP synthesized using molarity ratio of 0.6M CaO: 0.4M H<sub>3</sub>PO<sub>4</sub> had characteristics resembling  $\beta$ -TCP (Sigma-Aldrich).

**Keywords:**  $\beta$ -Tricalcium phosphate; dissolution precipitation; green mussel shell; whitlockite

### INTRODUCTION

Bone is hard tissues that can regenerate due to bone loss and damage caused by trauma, infection, tumors, resection, and bone abnormalities.<sup>1</sup> Bone defects can be overcome by surgical procedures, or bone grafting.<sup>2</sup> According to the source of material, bone grafts are divided into four types: autograft, allograft, xenograft, and alloplastic graft.<sup>3</sup> Alloplastic grafts are the only type of bone graft that comes from synthetic materials such as ceramics and polymers.<sup>4</sup>  $\beta$ -tricalcium phosphate is a commonly used alloplastic graft material and has a biodegradable level that corresponds to the rate of bone growth. Meanwhile, because hydroxyapatite has high stability in the bones, it is difficult to be absorbed by the body and will remain in the body for a long time.<sup>5</sup>

$\beta$ -TCP can be synthesized by reacting calcium and phosphate compounds. Natural materials such as clam shells can be used as a source of calcium and phosphoric acid, from which phosphate is derived.<sup>6</sup> Based on statistical data from the Ministry of Marine Affairs and Fisheries, shellfish production in Indonesia reached 148,381.02 tons in 2020.<sup>7</sup> One shellfish species with high economic value in Indonesia is green mussels. Green mussel shells contain calcium carbonate of 95-99%, so they can be used as a source of calcium. They also have a higher calcium content than other types of shells, such as kepa shells and abalone shells.<sup>8,9</sup> There are several methods that can be used for calcium phosphate synthesis: dissolution precipitation, sol-gels, hydrothermal, and various emulsions.<sup>10,11</sup>

The dissolution precipitation method is widely used today because this method is relatively cheaper, does not use organic solvents, and the process is easier and faster than other methods.<sup>12</sup>

In dentistry, bone graft material can be used for periodontal regeneration therapy, guided bone regeneration procedures before implant placement, and to fill bone defects caused by peri-implantitis.<sup>13,14</sup> The use of bone graft material is becoming increasingly popular. However, the price of bone graft material in Indonesia is expensive and not affordable for the lower to the middle class. Therefore, it is necessary to utilize natural materials and efficient methods to synthesize bone graft material. This study aims to determine the characteristics of  $\beta$ -TCP synthesized from calcium sources of green mussel shells and phosphoric acid solutions with variations in molarity to produce optimal  $\beta$ -TCP.

## MATERIALS AND METHODS

The samples for this study were green mussel shells as source of calcium and phosphoric acid solution, from which phosphate was derived, to fabricate  $\beta$ -TCP. Sample preparation began with cleaning the green mussel shells to remove dirt and mud. The samples were then left to dry at

room temperature for 24 hours and heated in an oven (UN55, Memmert, Büchenbach, Germany) for 5 hours at 110 °C. The shells were ground into powder and sieved to achieve 0.35-0.6 mm size. Furthermore, the powder was characterized using the XRD (Rigaku Minifex, Tokyo, Japan) to determine the formation of  $\text{CaCO}_3$ . Sintering was performed using a furnace (Ney Vulcan D130, Merck Dentsply, Woodbridge, Ontario, Canada) at 1,000 °C for 5 hours with an increase in temperature of 10 °C /min to convert  $\text{CaCO}_3$  to CaO.

CaO powder was reacted to phosphoric acid solution with four types of molarity ratios of CaO and  $\text{H}_3\text{PO}_3$  (0.6 M and 0.4 M; 1.2 M and 0.8 M; 1.8 M and 1.2 M; 2.4 M and 1.6 M). Ca/P ratio of 1.50 was required to form  $\beta$ -TCP (Table 1). However, before the CaO was immersed into the  $\text{H}_3\text{PO}_4$  solution, the phosphoric acid (85% w/w) solution was diluted by adding aquadest to the solution to obtain the appropriate molarity (Table 2). Dissolution precipitation method was carried out using a magnetic stirrer at 300 rpm at a temperature of 80 °C for 1 hour. The precipitate was then filtered and dried at 37 °C for 24 hours. Furthermore, the samples were sintered at 1,000 °C for 3 hours with an increase in temperature

**Table 1.** The composition of calcium and  $\text{H}_3\text{PO}_4$  solution

Comparison of CaO : $\text{H}_3\text{PO}_4$	Massa CaO (gr)	Volume $\text{H}_3\text{PO}_4$ (ml)
0.6 M : 0.4 M	3.36	100
1.2 M : 0.8 M	6.72	100
1.8 M : 1.2 M	10.08	100
2.4 M : 1.6 M	13.44	100

**Table 2.** pH of  $\text{H}_3\text{PO}_4$  solution after been diluted

$\text{H}_3\text{PO}_4$		Volume of aquades (ml)	pH
Molarity (M)	Volume (ml)		
0.4 M	2.7	97.3	1.4
0.8 M	5.4	94.6	1.1
1.2 M	8.1	91.9	1.0
1.6 M	10.8	89.2	0.7

of 5 °C/min. The  $\beta$ -TCP samples were subjected to characterization tests using XRD, SEM, and FTIR and compared with  $\beta$ -TCP (Sigma-Aldrich) as standard. Ethical approval was not applicable for this article since the experiment did not involve humans or animals. This experiment used the waste of green mussels.

## RESULTS

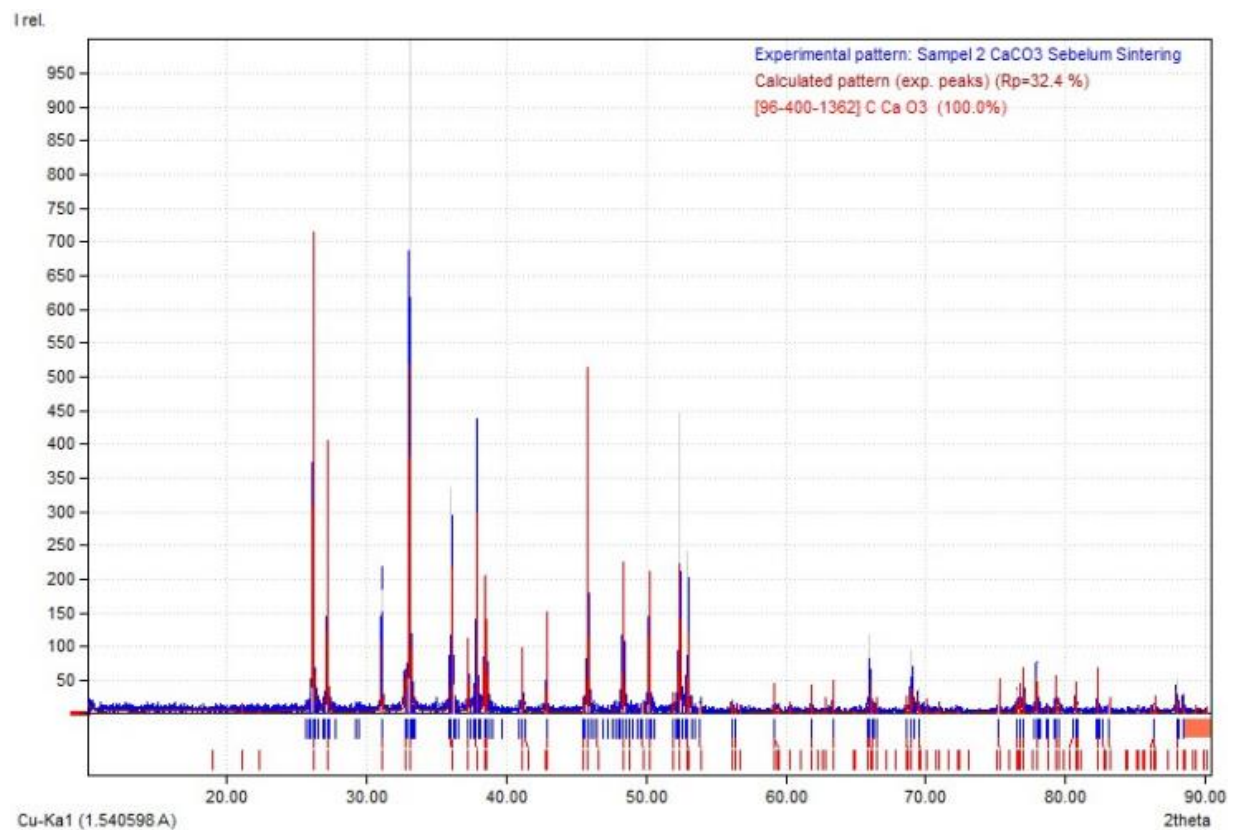
Figure 1 shows that green mussel shell powder contains calcium carbonate ( $\text{CaCO}_3$ ). Following sintering, CaO powder was produced from green mussel shell powder, as shown in Figure 2.

Samples produced after sintering were subjected to XRD characterization tests and compared with the characteristics of  $\beta$ -TCP (Sigma-Aldrich) powder. The graph of the results of the XRD characterization of  $\beta$ -TCP can be seen in Figure 3 and Table 3.

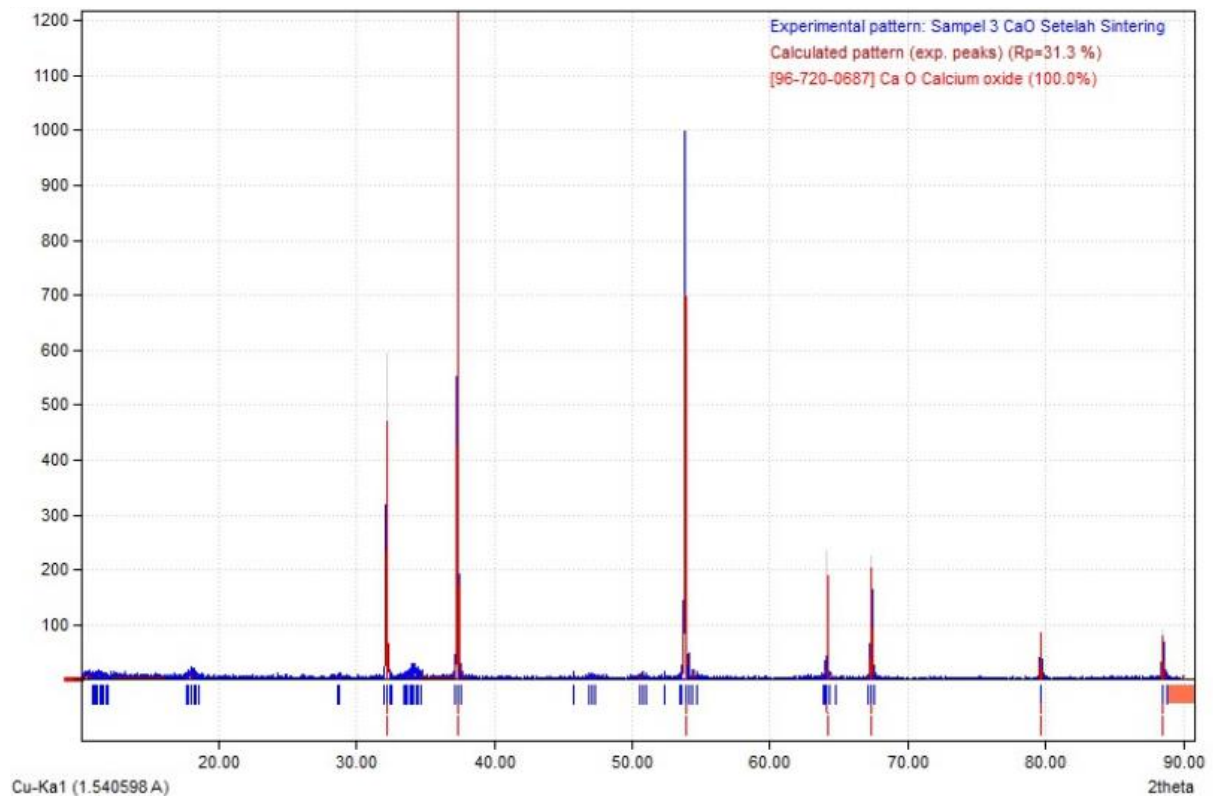
The FTIR test was performed to identify the chemical functional groups of the samples. Characterization with FTIR (Prestige 21, Shimadzu, Japan) was performed at a frequency range of 400-4000  $\text{cm}^{-1}$  to see the molecular bonds of  $\beta$ -TCP. The spectra of the FTIR characterization results can be seen in Figure 4.

**Table 3.** The peaks of the XRD results

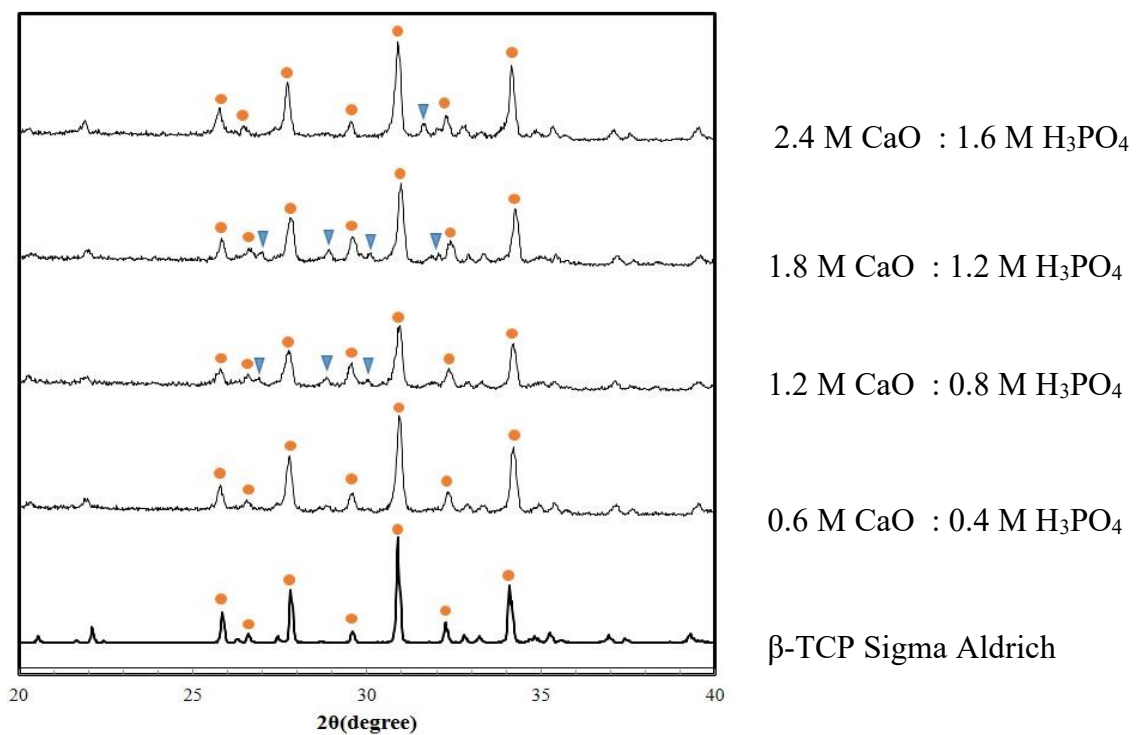
Peak	$2\theta$ (degrees)				
	$\beta$ -TCP <i>Sigma Aldrich</i>	$\beta$ -TCP 0.6 M : 0.4 M	$\beta$ -TCP 1.2 M : 0.8 M	$\beta$ -TCP 1.8 M : 1.2 M	$\beta$ -TCP 2.4 M : 1.6 M
1	27.79	28.15	28.18	27.98	27.89
2	31.03	31.39	31.39	31.19	31.14
3	34.37	34.69	34.71	34.56	34.48



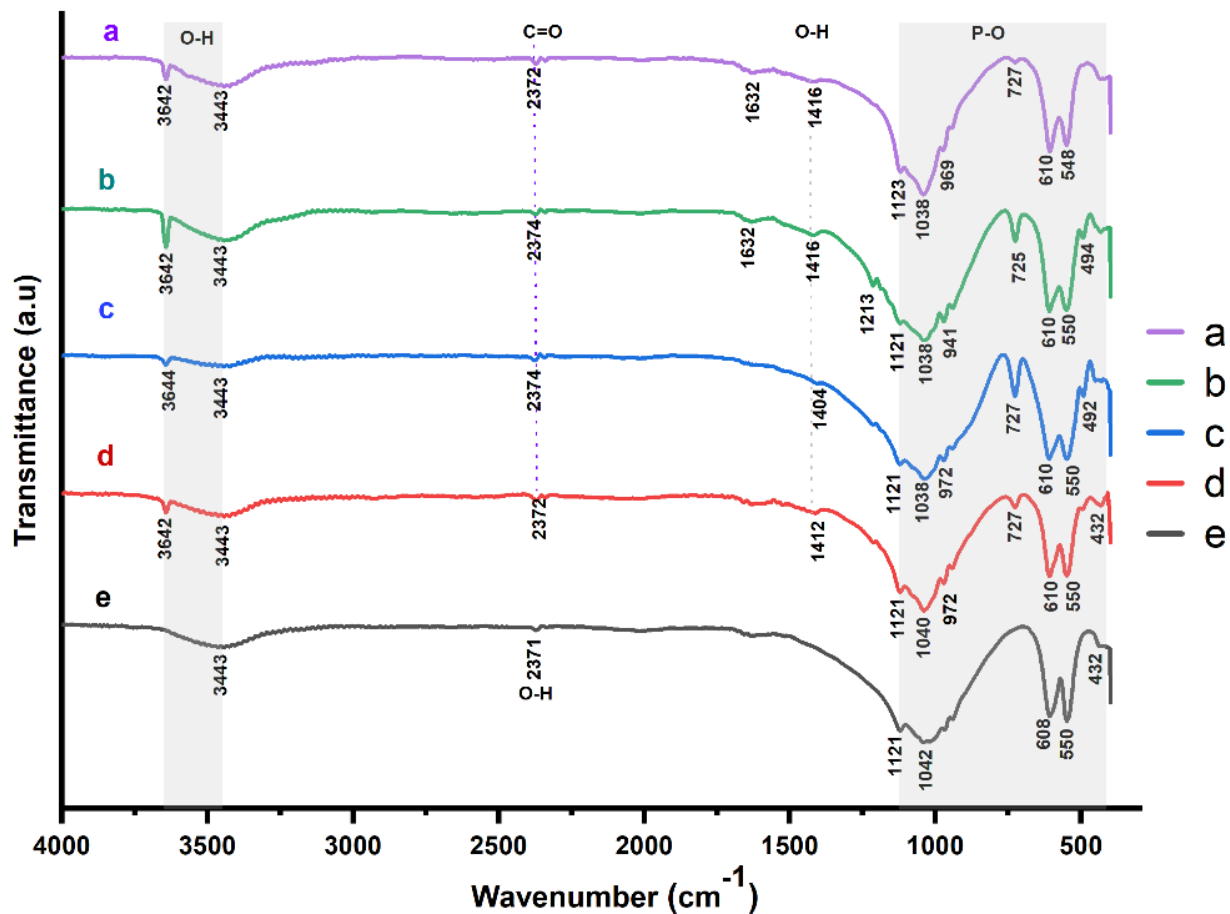
**Figure 1.** XRD graph of green mussel shell powder



**Figure 2.** XRD graph of CaO powder from green mussel shells



**Figure 3.** XRD characterization graph of  $\beta$ -TCP samples synthesized from green mussel shells with molarity ratio of CaO and  $H_3PO_4$  of 0.6 M and 0.4 M; 1.2 M and 0.8 M; 1.8 M and 1.2 M; 2.4 M and 1.6 M. Sigma Aldrich  $\beta$ -TCP powder. The sign (●) indicates the formation of a  $\beta$ -TCP diffraction pattern, while the sign (▼) indicates the formation of a whitlockite diffraction pattern



**Figure 4.** Graph of  $\beta$ -TCP FTIR spectrum analysis results TCP (a) 2.4 M CaO : 1.6 M  $H_3PO_4$ ; (b) 1.8 M CaO : 1.2 M  $H_3PO_4$ ; (c) 1.2 M CaO : 0.8 M  $H_3PO_4$ ; (d) 0.6 M CaO : 0.4 M  $H_3PO_4$ ; (e)  $\beta$ -TCP Sigma Aldrich

**Table 4.** The range of particle sizes and micropores formed in the samples

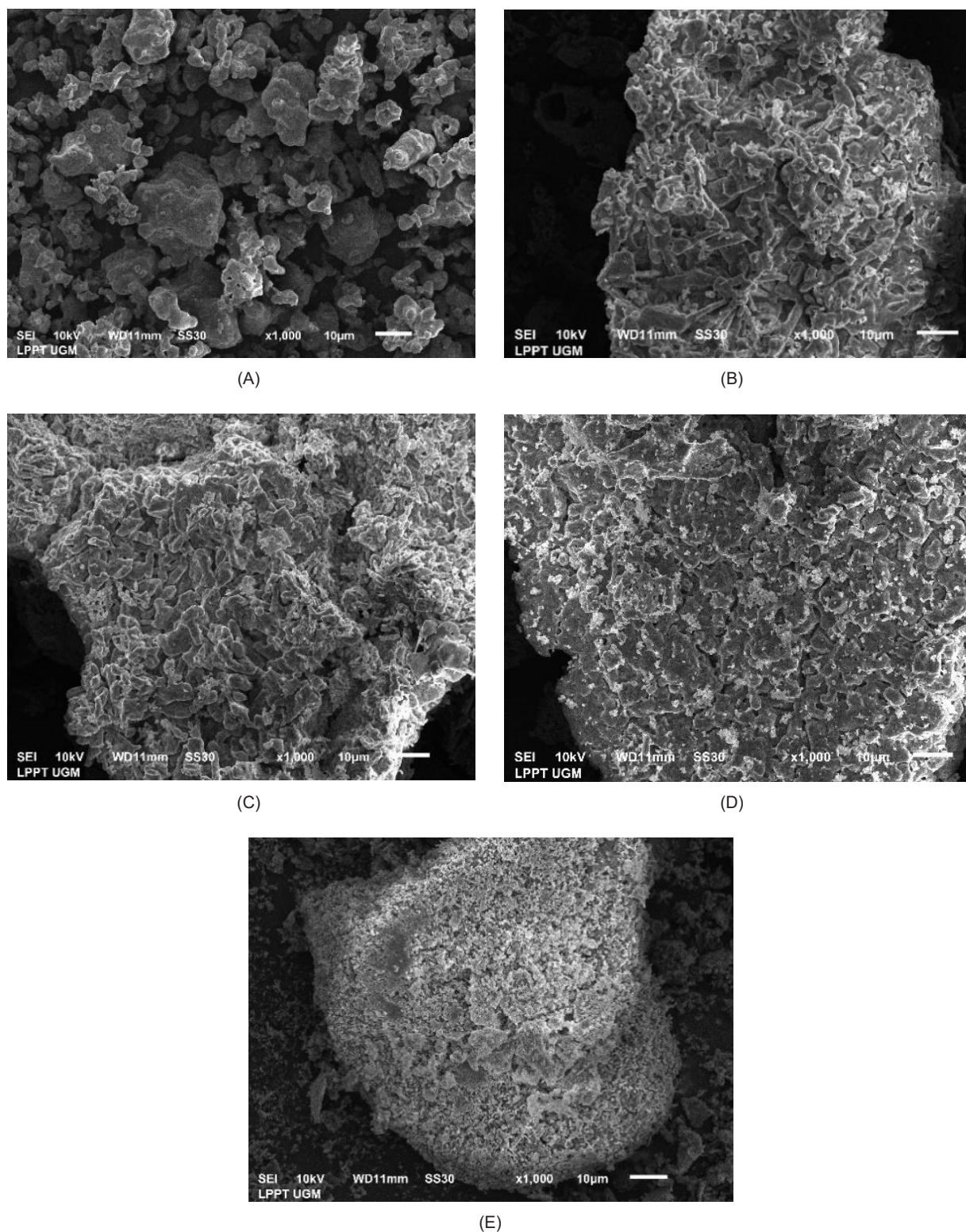
Sample	Range of particle sizes ( $\mu\text{m}$ )	Range of micropores ( $\mu\text{m}$ )
$\beta$ -TCP Sigma Aldrich	1.0 – 5.7	0.4 – 10.8
0.6 M CaO : 0.4 M $H_3PO_4$	0.8 – 5.6	0.2 – 10.0
1.2 M CaO : 0.8 M $H_3PO_4$	0.6 – 4.2	0.4 – 7.1
1.8 M CaO : 1.2 M $H_3PO_4$	0.4 – 5.4	0.3 – 4.1
2.4 M CaO : 1.6 M $H_3PO_4$	0.2 – 2.7	0.2 – 3.1

SEM characterization was carried out to determine the morphological shape and size of the pores that could give  $\beta$ -TCP osteoinductive properties. Figure 5-7 and Table 4 show the result of SEM characterization with a magnification of 1.000x, 3.000x, and 5.000x.

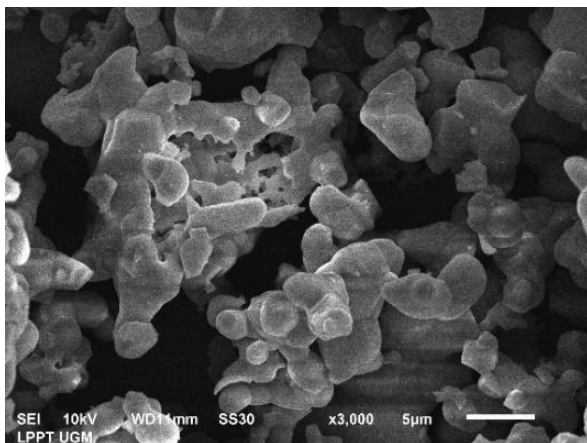
## DISCUSSION

Green mussel shell powder contains calcium carbonate ( $CaCO_3$ ), which can be used as a source of calcium to synthesize  $\beta$ -TCP. Liemawan's research revealed that green mussel shells have  $CaCO_3$  content of 95.7%.<sup>15</sup>  $CaCO_3$  has 3 phases: (a) calcite, a stable phase at room temperature and can form at low temperatures, (b) aragonite, a metastable phase and can form at high temperatures, (c) vaterite, a metastable phase at room temperature and can form at high saturation levels.<sup>16,17</sup> Ismail's research found that green mussel shell powder crystals are rich in  $CaCO_3$  as an aragonite phase.<sup>18</sup>

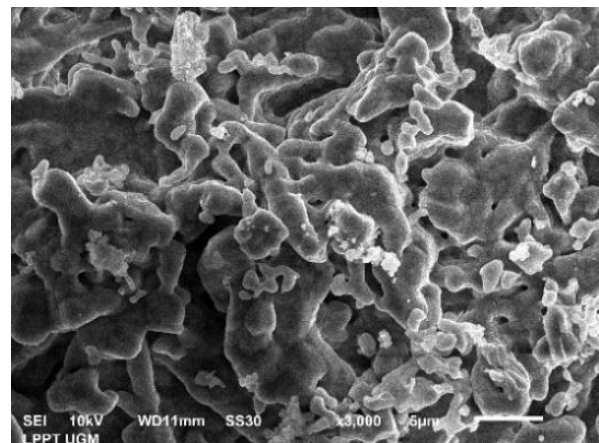
Green mussel shell powder was sintered at 1,000 °C for 5 hours to remove organic components and convert  $CaCO_3$  into CaO by releasing carbon dioxide ( $CO_2$ ).<sup>19,20</sup>  $CaCO_3$  is a stable phase with low solubility, so the reaction



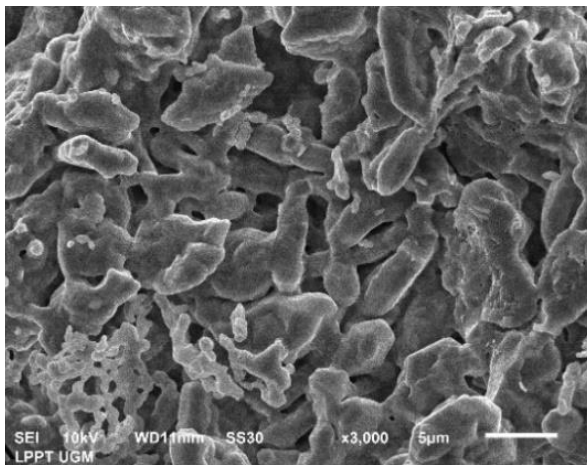
**Figure 5.** SEM characterization test results of sample  $\beta$ -TCP with 1.000x magnification. (A)  $\beta$ -TCP sigma aldrich; (B) 0.6 M CaO : 0.4 M H<sub>3</sub>PO<sub>4</sub>; (C) 1.2 M CaO : 0.8 M H<sub>3</sub>PO<sub>4</sub>; (D) 1.8 M CaO : 1.2 M H<sub>3</sub>PO<sub>4</sub>; (E) 2.4 M CaO : 1.6 M H<sub>3</sub>PO<sub>4</sub>



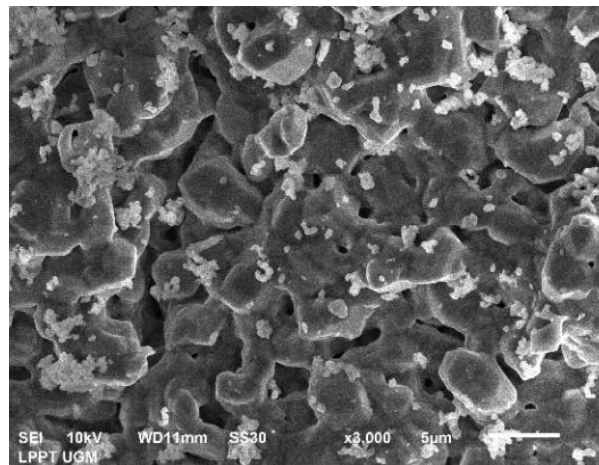
(A)



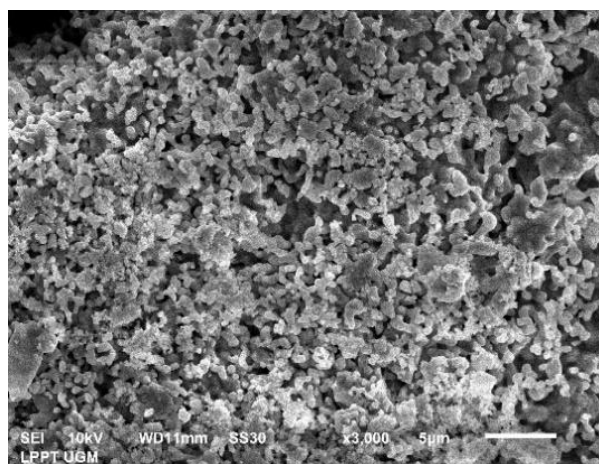
(B)



(C)

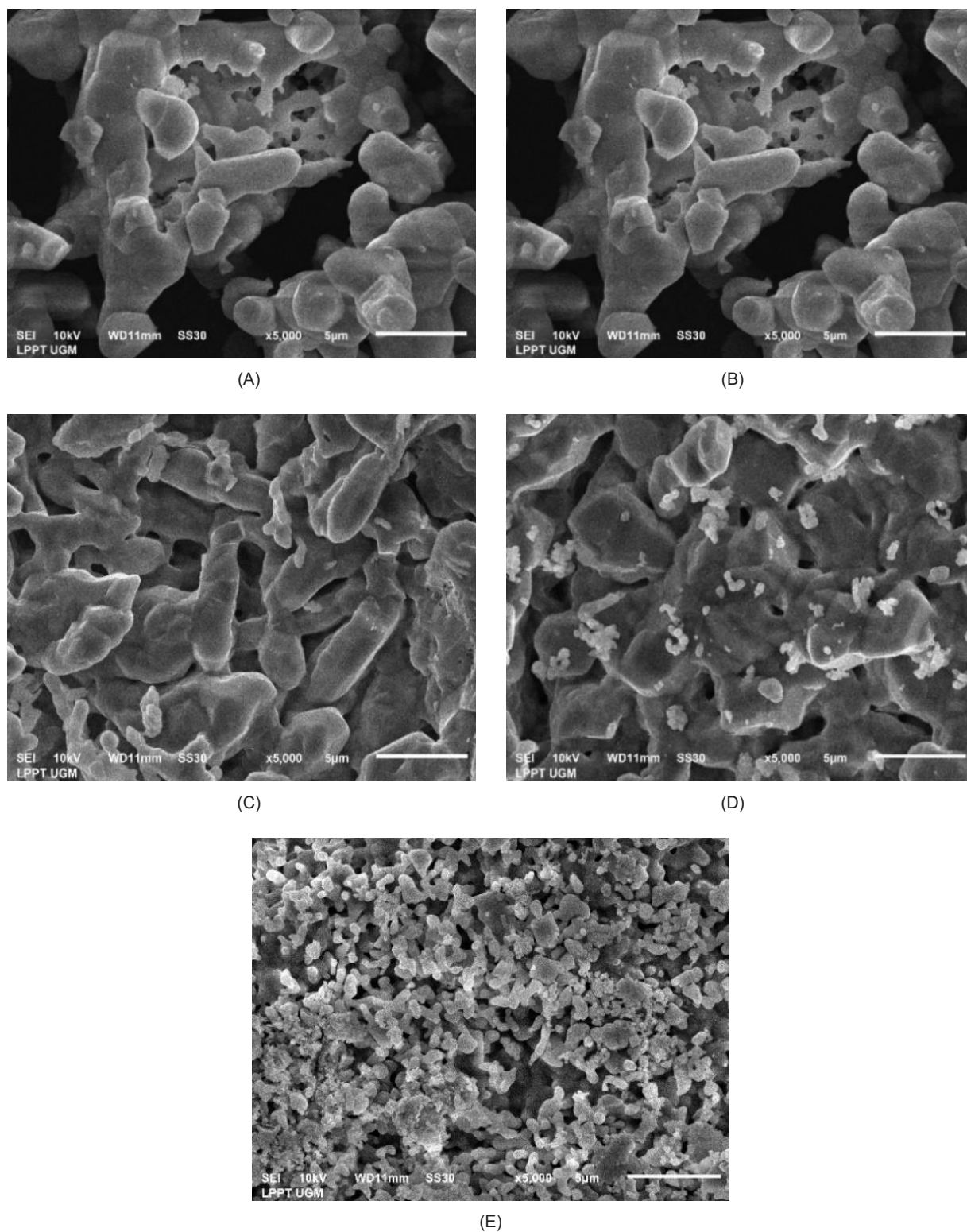


(D)



(E)

**Figure 6.** SEM characterization test results of sample  $\beta$ -TCP with 3.000x magnification. (A)  $\beta$ -TCP sigma aldrich; (B) 0.6 M CaO : 0.4 M  $H_3PO_4$ ; (C) 1.2 M CaO : 0.8 M  $H_3PO_4$ ; (D) 1.8 M CaO : 1.2 M  $H_3PO_4$ ; (E) 2.4 M CaO : 1.6 M  $H_3PO_4$



**Figure 7.** SEM characterization test results of sample  $\beta$ -TCP with 5.000x magnification. (A)  $\beta$ -TCP sigma aldrich; (B) 0.6 M CaO : 0.4 M  $H_3PO_4$ ; (C) 1.2 M CaO : 0.8 M  $H_3PO_4$ ; (D) 1.8 M CaO : 1.2 M  $H_3PO_4$ ; (E) 2.4 M CaO : 1.6 M  $H_3PO_4$



between calcium and phosphate compounds are more complex and longer.<sup>21</sup> Therefore, in our study  $\text{CaCO}_3$  was converted to  $\text{CaO}$  to accelerate the dissolution precipitation reaction. Green mussel shell powder that had been sintered produced a slightly greenish-white powder.

Synthesis of  $\beta$ -TCP was carried out using the dissolution precipitation method, forming a precipitate in a solution due to a chemical reaction. This precipitate is formed when the concentration of dissolved ions has reached the solubility limit and forms a salt compound.<sup>22</sup> The material for establishing  $\beta$ -TCP is  $\text{CaO}$  synthesized from green mussel shells and phosphoric acid solution, which has a Ca/P ratio used of 1.50.<sup>23,24</sup>

This precipitate produced white and fine powder. The white color of the sample indicated calcium content in the compound. Sintering process was then performed with a temperature of 1,000 °C to fuse two different substances, remove  $\text{CO}_2$  elements, and determine the mechanical properties and porosity of the resulting sample.<sup>25,26</sup> The success of  $\beta$ -TCP synthesis from green mussel shells was confirmed by conducting characterization tests using XRD, FTIR and SEM.

The results of the XRD analysis showed that the composition of  $\beta$ -TCP synthesized from green mussel shells was qualitatively similar to  $\beta$ -TCP (Sigma-Aldrich). The  $\beta$ -TCP graph pattern with a molarity ratio of 0.6 M  $\text{CaO}$  : 0.4 M  $\text{H}_3\text{PO}_4$  was the most similar to  $\beta$ -TCP (Sigma-Aldrich). The results of the XRD analysis found that whitlockite phase ( $\text{Ca}_{18}\text{Mg}_2(\text{HPO}_4)_2(\text{PO}_4)_{12}$ ), a type of calcium phosphate material, was also formed. Whitlockite had the same XRD pattern and Ca/P molarity ratio close to  $\beta$ -TCP, which was 1.43. The different Ca/P ratio and solubility could release different free  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{P}^+$  ions. Whitlockite continuously released  $\text{Mg}^{2+}$  and  $\text{P}^+$  ions at higher levels than  $\beta$ -TCP under physiologically relevant conditions. In the research by Jang et al., the synthesis of whitlockite can be carried out by the dissolution precipitation method, namely by reacting the compounds  $\text{Ca}(\text{OH})_2$ ,  $\text{Mg}(\text{OH})_2$ , and  $\text{H}_3\text{PO}_4$  at 70 °C under acidic conditions.<sup>27-29</sup> Based on the XRD characterization results, this whitlockite has a

chemical structure of  $\text{Ca}_9(\text{Fe}_{0.63}\text{Mg}_{0.37})\text{H}_{0.37}(\text{PO}_4)_{17}$ . Magnesium ( $\text{Mg}^{2+}$ ) and iron ( $\text{Fe}^{2+}$ ) ions may come from green mussel shells. According to Ismail's research, light elements produced from oxidation processes, such as oxygen and carbon, could possibly bind carbonates. In this case, various additional Ca-, Mg-, Mn-, and Fe-rich carbonate minerals can be found in green mussel shells.<sup>18</sup> Research by Su Yeon Kwon showed that whitlockite could be used as a bone graft material due to its conductivity and biocompatibility properties comparable to hydroxyapatite and  $\beta$ -TCP,<sup>27,30</sup> considering whitlockite has magnesium ions which can act as a cofactor for many enzymes and stimulate new bone growth, adhesion, and proliferation of osteoblastic cells to increase biocompatibility and bone formation.<sup>28,31</sup>

In the FTIR analysis spectra, the phosphate groups' sharp peaks indicate a better crystallinity growth to improve the quality of  $\beta$ -TCP. The phosphate group has four vibrational modes consisting of stretching symmetry ( $\nu_1$ ), bending symmetry ( $\nu_2$ ), stretching asymmetry ( $\nu_3$ ), and bending asymmetry ( $\nu_4$ ).<sup>26,32</sup>  $\beta$ -TCP (Sigma-Aldrich) and  $\beta$ -TCP synthesized from green mussel shells have three vibration modes: bending symmetry, stretching asymmetry, and bending asymmetry. In the FTIR analysis results, hydroxyl groups (OH) indicate that the sample still contains  $\text{H}_2\text{O}$  (water).<sup>33</sup>

In addition, the results of the FTIR characterization of the study sample found peaks at wave numbers 727  $\text{cm}^{-1}$  dan 725  $\text{cm}^{-1}$ , which were not present in  $\beta$ -TCP (Sigma-Aldrich) sample. A study by Ruiz-Aguilar et al. reported that this group is the residual amount of  $\text{P}_2\text{O}_7$  resulting from an incomplete transformation process from TCP to  $\beta$ -TCP phase. Several studies have found that differences in Ca/P ratio lead to the formation of  $\beta$ -calcium pyrophosphate ( $\beta\text{-Ca}_2\text{P}_2\text{O}_7$ ) as the residual phase in the TCP synthesis process.<sup>34</sup>  $\beta\text{-Ca}_2\text{P}_2\text{O}_7$  is a calcium phosphate material that has the potential as a bone graft material with a Ca/P ratio 1.00.  $\beta\text{-Ca}_2\text{P}_2\text{O}_7$  has biocompatible, biodegradable properties and can be absorbed biologically.<sup>35</sup> This indicates that  $\beta\text{-Ca}_2\text{P}_2\text{O}_7$  is not

toxic to the human body and has almost similar properties to  $\beta$ -TCP.

The results of SEM characterization showed that the  $\beta$ -TCP sample consisted of small irregularly shaped particles, and some of them were bound together like aggregates. Particle size and crystallinity are essential factors in making biomaterials because the particle size affects the properties of calcium phosphate, such as mechanical strength, degradation rate, protein adsorption, and biocompatibility.<sup>36,37</sup> The result of SEM characterization (Table 4) suggests that the greater the concentration value of CaO and  $H_3PO_4$  in the sample, the smaller the particle size. The SEM images also showed the presence of micropores with varying shapes and size ranges. Bohner's research found that micropores positively affect the biological response of  $\beta$ -TCP to increase resorption and osteoinduction. In addition, the formation of pores in  $\beta$ -TCP can also help the circulation and exchange of body fluids, nutrient supply, osteoblast penetration, ion diffusion, and vascularization.<sup>25</sup>

## CONCLUSION

This study has shown that  $\beta$ -tricalcium phosphate could be synthesized by reacting calcium from green mussel shells with a phosphoric acid solution. Based on the XRD, FTIR, and SEM results, samples with a molarity ratio of 0.6 M CaO : 0.4 M  $H_3PO_4$  had the characteristics that were more similar to  $\beta$ -TCP (Sigma-Aldrich) compared to other molarity comparisons.

## CONFLICT OF INTEREST

The authors declare no conflict of interest with the data contained in the manuscript.

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