

The Effect of Poly (Vynil Pyrrolidine) (PVP) Added in Variation of Ca²⁺ and PO₄³⁻ Concentration in Microbial Cellulose-Hydroxyapatite Composite As Scaffold For Bone Healing

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ABSTRACT

In Indonesia, the traffic accident causing 6 million people suffered injuries, particularly injuries fractures. 46.2% incidence of fractures occur in the lower extremities, 25% of them dying, 45% had a physical disability, 15% experienced psychological pressure and only 10% were healed well. Implant (graft) are used to support and accelerate the healing process of broken bones (bone healing). This study was done to make microbial cellulose-hydroxyapatite scaffold as a candidate for bone healing. Microbial cellulose obtained from culturing *Acetobacter xylinum* is used as a matrix and hydroxyapatite as a filler that is synthesized using the method of immersion in a solution of CaCl₂ and KH₂PO₄, to increase the formation of apatite crystals, added polyvinyl Pyrrolidine (PVP). Scaffold synthesized using methods of freeze dried. Formation of composites varied in the concentration of Ca²⁺ and PO³⁻ of 25:125; 50:100; 75:75; 100:50 mM. The samples were then characterized using FTIR spectroscopy which shows the phosphate groups and the carbonate indicates the formation of hydroxyapatite in the eighth sample. Furthermore, to determine the morphology and identify the elements in the scaffold used SEM-EDAX, it was found that the pore formed measuring about 150-300 μm and obtained ratio of Ca / P best on microbial cellulose scaffold-PVP-hydroxyapatite with a variation of the concentration of Ca²⁺ and PO₄³⁻ of 100 : 50 mM is equal to 0.6046 with an average degradation rate of 18.617% and the percentage of porosity contained in the sample amounted to 88.4%. This proves that microbial cellulose scaffold-PVP-hydroxyapatite with a variation of the concentration of Ca²⁺ and PO₄³⁻ of 100:50 mM potential as a candidate for bone healing.

Keywords: bone healing, scaffold, selulosa mikrobial, hidroksiapatit, poly(vynil pyrrolidine), freeze dried

INTRODUCTION

Fracture is a condition where there is discontinuity in the bone and is determined according to the type and extent, the fracture can be caused by trauma, both total and partial (Smeltzer and Bare, 2006). Signs and symptoms of broken bones are deformity, swelling, pain, functionolesa (loss of

function), abnormal mobility and changes in neurovascular (Black and Hawks, 2009).

The overall fracture incidence occurs around 11.3% in 1,000 events per year, of which 11.67% in 1,000 occurrences per year occur in males, while in females it occurs around 10.65% in 1,000 events per year (Bucholz et al., 2006). In Indonesia, around 6

million people were injured due to traffic accidents and also killed nearly 1.3 million people worldwide (MOH, 2007; WHO, 2011). Based on police reports, accidents in Indonesia increased by 6.72% from 57,726 incidents in 2009 to 61,606 incidents in 2010 or 168 incidents occurred every day and 43.15% died (WHO, 2011). Of the fracture events in Indonesia 46.2% of the events were fractures in the lower extremities, then found 25% of fracture sufferers died, 45% experienced physical disabilities, 15% experienced psychological distress and 10% experienced healing well (MOH, 2007).

Management of fractures is by recognizing, reducing (retaining), retaining (maintaining) and rehabilitation (Helmi, Z.N., 2011; Bucholz et al., 2006). The principle of treating fractures is to return the position of the broken bone to its original position (repositioning) and maintain that position during the healing process of the bone (immobilization) (Salter, R.B., 1999). In the repositioning process using the method of Minimal Invasive Plate Osteosynthesis (MIPO), a tool that is used is internal fixation, there are two principles in the installation of this fixation, namely to restore broken bone to its original place and also to support bone healing with rigid stabilization in the form of compression. This compression is useful to assist in the bone healing process (bone healing) in the primary where osteon (hard bone tissue) will be formed due to compression between the surface of bone fragments. Internal fixation (implants) commonly used are screws and plates, to prevent complications due to the type of

material used on the screw and plate implants in the form of bone graft (Djoko, S.I., 2008).

The use of bone graft in Indonesia is increasing from year to year both in the field of orthopedics and periodontal. Graft is useful as a substitute for damaged bone, there are three types, namely autograft, allograft, and xenograft. Autograft is a bone graft originating from the patient himself, allograft is a graft that comes from other people both living and corpse, xenograft is a graft derived from animals. Lack of autograft is limited, both from the number of donors and the bone itself.

Whereas allograft and xenograft because they are not from the patient tend to cause autoimmune reactions when transferred to a larger patient body (Darmawan and Yessi, 2008). Because of these constraints, alternatives arise in replacing the material from the implant with synthetic material, of course the implant made must resemble the actual bone mineral composition (Dahlan and Dewi, 2013). Implants (graft) that are used to support bone healing (bone healing) must have certain properties, which have properties such as human bones, easily obtained, biocompatible, bioactive, and non-toxic (Riyani, 2005).

Hydroxyapatite (HA) is a matrix of mineral components in bone, with the chemical formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ being stable calcium phosphate under normal physiological conditions. HA is a bioceramics that has biocompatible, bioactive, osteoconductive, non-toxic, and non-inflammatory properties (Rozita et al, 2011). However, when HA is used alone, HA has no mechanical strength and cannot withstand pressure (Hutchens et al.,

2004). For this reason, it is required a combination of materials (composites) containing HA so that later the mechanical strength produced is the same as the actual bone and is resistant to pressure, the material chosen to be combined with HA in this study is microbial cellulose.

Cellulose is an abundant component contained in plant cells. Cellulose can be synthesized by various organisms from multicellular and unicellular plants and bacteria. There are some bacteria that are known to produce cellulose, but only gram-negative bacteria. *Acetobacter xylinum* which can produce cellulose in sufficient quantities to meet market needs. Cellulose produced by bacteria is called microbial cellulose or biosellulose (Darmawan, 2009).

The advantage of microbial cellulose is the purity of its compounds, because if we take cellulose from plants, there are lignin and hemicellulose contents that are difficult to remove. Microbial cellulose has biocompatible and biodegradable properties for tissues (Darmawan, 2009). Microbial cellulose also has a high surface activity because it consists of fibril fibers, so microbial cellulose can be a good material if used as a matrix on composites (Yamane et al., 2004). According to Yamanaka et al., (1989) microbial cellulose has a young modulus of 16-18 GPa and can be increased to 30 GPa with further purification, so microbial cellulose can be used as a hydroxyapatite matrix because Young human bone modulus ranges from 12-24 GPa (Yamanaka et al., 1989).

The fibers produced by microbial cellulose are around 100 nm in diameter and can form

clearly 3D networks. The mechanical properties of these structures depend on the tissue formed. Microbial cellulose tissue can be used as a gel-like, hot-pressed material as a dry sheet or freeze-dried. Biocompatible microbial cellulose makes cellulose used as a scaffold in tissue engineering applications. Some of the characteristics that must be possessed by a scaffold are that they must have an appropriate pore scale to support tissue integration and vascularization, surface chemical properties

appropriate to support cell attachment, differentiation and proliferation, adequate mechanical properties (no more or no less) and must be made of materials that can be controlled biodegradation and bioresorption so that bone tissue can replace the scaffold later (Fernando et al. , 2012).

Based on research conducted by Windarti et al., (2006), hydroxyapatite production was carried out in the bacterial cellulose matrix by varying the concentration of Ca^{2+} and PO_4^{3-} ions from CaCl_2 and KH_2PO_4 sources of 25: 125mM, 50: 100mM, 75: 75mM, and 100: 50mM in which the Ca / P ratio is obtained in the range of 0.04-0.05 which the magnitude of the Ca / P ratio still does not meet the standards. Furthermore, in a study conducted by Yin et al., (2011), a composite between microbial cellulose and hydroxyapatite was made using a biomimetic method by immersing microbial cellulose and HA composites into Poly (Vynil Pyrrolidone) (PVP) for 2 days and CaCl_2 for 3 days then into the Simulated Body Fluid (SBF) for 5 days and 7 days to induce the formation of apatite crystals. From these studies it was found that

microbial-hydroxyapatite cellulose composites immersed in PVP contained more apatite crystals, therefore PVP was indicated to have the ability to encourage apatite nucleation and increase the level of mineralization. In addition it was also found that the best Ca / P results of 1.59 on microbial-HA-PVP cellulose composites, but these results are still not approaching the standard of the actual bone, because according the ideal content of hydroxyapatite literature is 39.9% Ca, 18.5% P and 3.38% OH with a Ca / P ratio of 1.67 (Chang, 1992).

In this research, a modification will be made on the making of microbial-HA-PVP cellulose composites by immersing microbial-PVP cellulose pellicles into CaCl_2 and KH_2PO_4 , soaking in CaCl_2 and KH_2PO_4 is carried out to form hydroxyapatite crystals in the pellicle. CaCl_2 and KH_2PO_4 were chosen as sources of calcium and phosphate in hydroxyapatite because the time needed for hydroxyapatite to be stored in microbial cellulose was shorter, each of which only required immersion for 18 hours (Windarti et al., 2006). Based on Windarti et al., (2006) research in which the results of the Ca/P ratio on microbial-HA cellulose composites still did not meet the standards, so to get a Ca/P ratio

that was in accordance with the standards in this study, variations in Ca^{2+} concentration were carried out. and $(\text{PO}_4)^{3-}$ (according to Windarti et al., 2006) where it is expected that the formed apatite crystals will increase with increasing Ca^{2+} concentration and $(\text{PO}_4)^{3-}$ during immersion. The presence of PVP is also expected to influence the formation of apatite crystals because in the study of Yin et al., (2011) it is indicated that PVP can encourage nucleation of apatite crystals.

Microbial cellulose composites and HA formed are then formed into scaffolds using the freeze dried method and then characterized to determine the characteristics of scaffold that will be applied for bone healing through several tests, namely functional group tests using Fourier Transform Infra Red (FTIR) to determine the constituent functional groups in the scaffold, test to determine morphology, pore size and identification of elements on the composite formed using a Scanning Electron Microscope (SEM)-Energy Dispersive X-ray (EDX) tool, degradation test using Simulated Body Fluid (SBF) to determine the ability of scaffold to be degraded in the body, the porosity test uses the liquid displacement method to determine the percentage of porous contained in the scaffold.

MATERIALS AND METHODS

Materials

Materials used in the study included 1 liter of coconut water, 150 ml of *Acetobacter xylinum*, 0.5% of the weight of sucrose used (5 grams) urea, 50 grams of sucrose, 1% of acetic acid as

much as 10 ml, CaCl_2 , KH_2PO_4 , *Poly(Vinyl Pyrrolidone)* (PVP) 0.05 grams, distilled water, and SBF solution.

Method

Material Preparation Stage

The materials used to make microbial-hydroxyapatite cellulose scaffold composites are 1 liter coconut water, 50 gram sucrose, 0.5% urea (5 gram), 1% acetic acid (10ml),

Acetobacter xylinum 20% (200ml), CaCl₂, KH₂PO₄, and Poly (Vynil Pyrrolidone) (PVP) 0.05 grams in 200 ml of water.

Making microbial cellulose matrix (Acetobacter xylinum)

The making of microbial cellulose matrix (Acetobacter xylinum) is done by preparing the tools and materials first. The tools needed are, pH indicator, erlenmeyer, measuring cup, mold / baking tray, pans, pipettes, digital scales, tongs and spatulas. The materials needed are coconut water, sucrose, urea, and culture of Acetobacter xylinum. After the tools and materials are collected, 1 liter of coconut water is prepared. Then weigh 50 grams of sucrose and 0.5% of urea (5 grams), then weighed sucrose and urea are mixed in 1 liter of coconut water in a heated

state. Add 1% acetic acid (10ml) to make a pH of 4. Stir in a solution consisting of water coconut, sucrose, and urea to homogeneous. After that, the homogeneous solution is allowed to stand for a moment at room temperature (27°C) with a pH check up to 4. Then add a culture of Acetobacter xylinum as much as 20% (200ml) and stir the solution until it is homogeneous. After the solution is homogeneous, the solution is poured into a mold container / baking pan and incubates at room temperature (27°C) for 7 days.

Manufacture of microbial cellulose scaffold composites-hydroxyapatite

The microbial cellulose matrix (Acetobacter xylinum) that was formed was made into two samples, the first sample was immersed in Poly (Vynil Pyrrolidone) (PVP) 0.05 gram in 200 ml distilled water for 7 days, and the second sample (control) was not immersed in Poly (Vynil Pyrrolidone) (PVP). After immersion in Poly (Vynil Pyrrolidone) (PVP) 0.05 gram in 200 ml of distilled water in the second sample, the next method was carried out, namely CaCl₂ immersion and KH₂PO₄ immersion for 18 hours

respectively, in this immersion the variation of Ca²⁺ and (PO₄)³⁻ concentrations was carried out. - i.e. 25: 125; 50: 100; 75:75; and 100: 50 mM, the method for immersion of CaCl₂ and KH₂PO₄ for 18 hours is expected to bind Ca²⁺ and (PO₄)³⁻ ions. After the sample is formed microbial-hydroxyapatite cellulose composite then freeze drying is carried out, the purpose of this freeze drying is that the material made can form a scaffold.

RESULTS AND DISCUSSION

Microbial-hydroxyapatite cellulose scaffold is made by biomimetic immersion method by immersing 1x1 cm microbial cellulose pellets in Poly (pyrene vynil) for 7 days and then soaking microbial-PVP cellulose pellets in CaCl₂ solution for 18 hours, then in KH₂PO₄ solution for 7 days and then soaking microbial-cellulose pellicle pellets into CaCl₂ solution for 18 hours, then in KH₂PO₄ solution for 7 days 18 hours to get apatite crystals. In the immersion of each of the microbial-PVP cellulose samples into CaCl₂ and KH₂PO₄ the

Cluster Test Results

The functional group testing on microbial-hydroxyapatite cellulose scaffold was carried out to identify the functional groups contained in the scaffold. At the time of testing, KBr powder is needed to provide background, KBr is mixed with the sample and crushed until smooth and then placed on a platinum pan

concentration variations were carried out by 25mM: 125mM, 50mM: 100mM, 75mM: 75Mm and 100mM: 50mM. Then the pellicle is formed into a scaffold using the freeze dried method. Microbial cellulose-PVP-hydroxapatite scaffold was then characterized by functional group tests using Fourier Transform Infra Red (FTIR), morphological tests using Scanning Electron Microscopy-Energy Disperse Xray (SEM-EDX), degradation test and porosity test.

which is then formed by pellets and pressed using mechanical hydraulics so that the sample becomes solid and can later be passed by infrared light. The test results are shown through the graph of the relationship between the wave number (cm⁻¹) and the transmittance value (%).

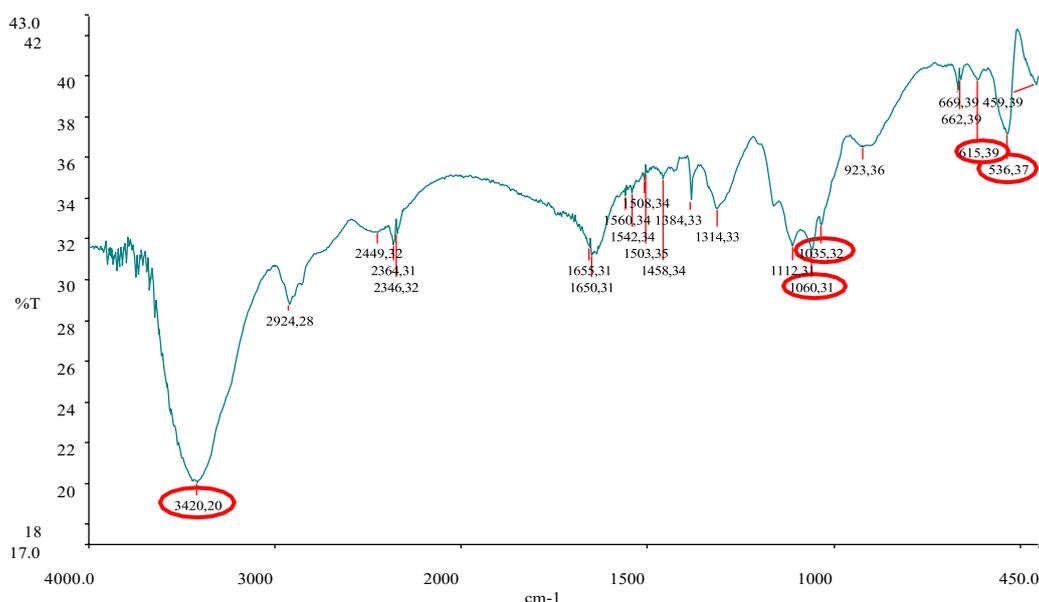


Figure 1 (a) FTIR Microbial-HA Cellulose Scaffold Spectra (25:125) (Control A)

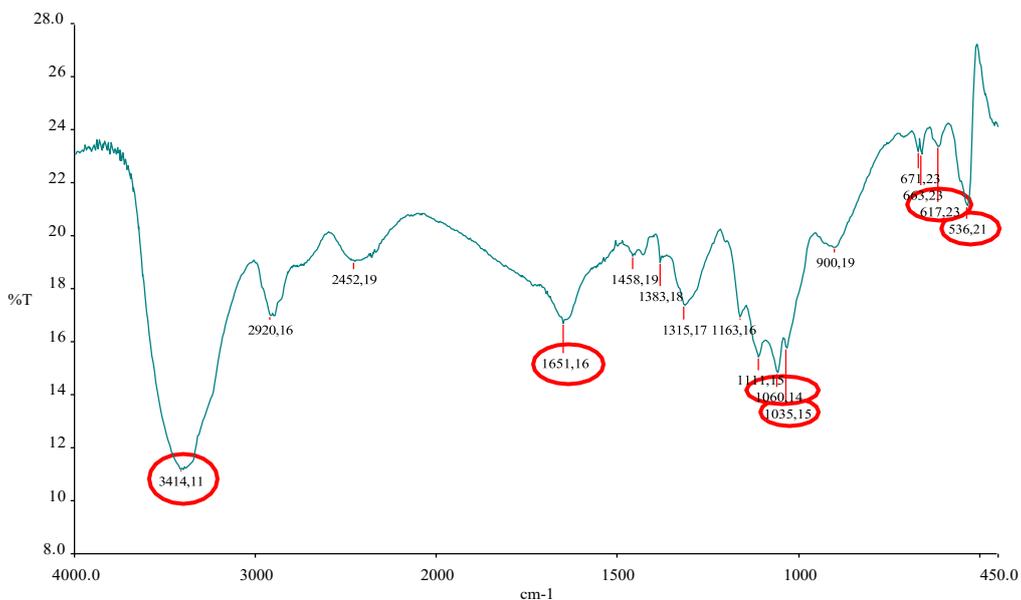


Figure 1 (b) FTIR-Microbial Cellulose-PVP-HA Scaffold Spectra (25:125)
(Sample A)

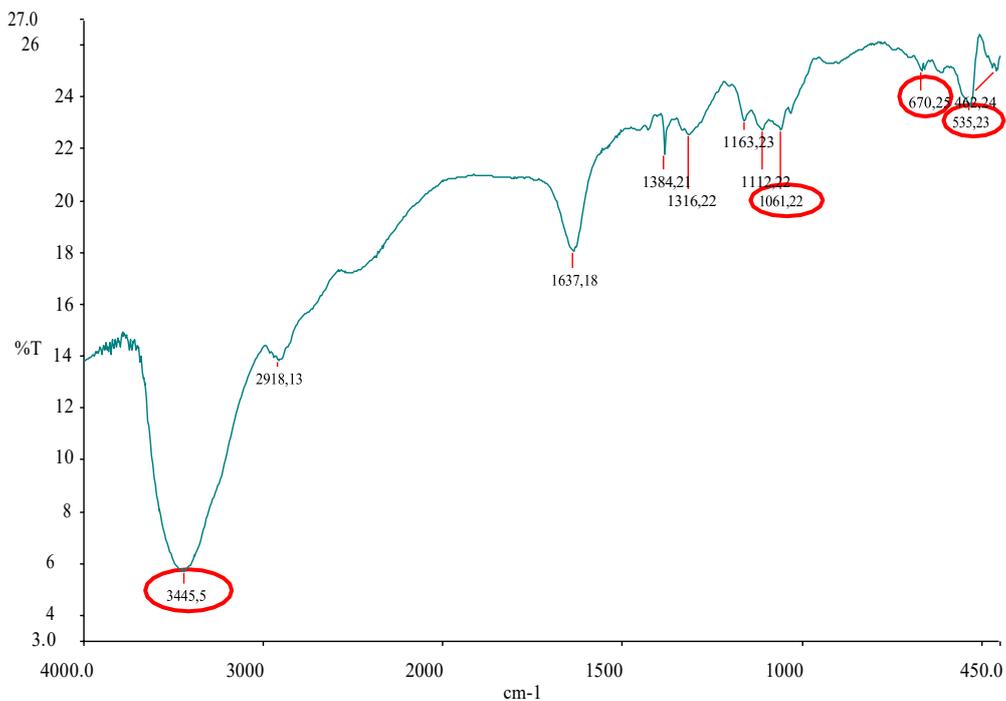


Figure 2. (a) Microbial-HA (50:100) FTIR Scaffold Cellulose Spectra
(Control B)

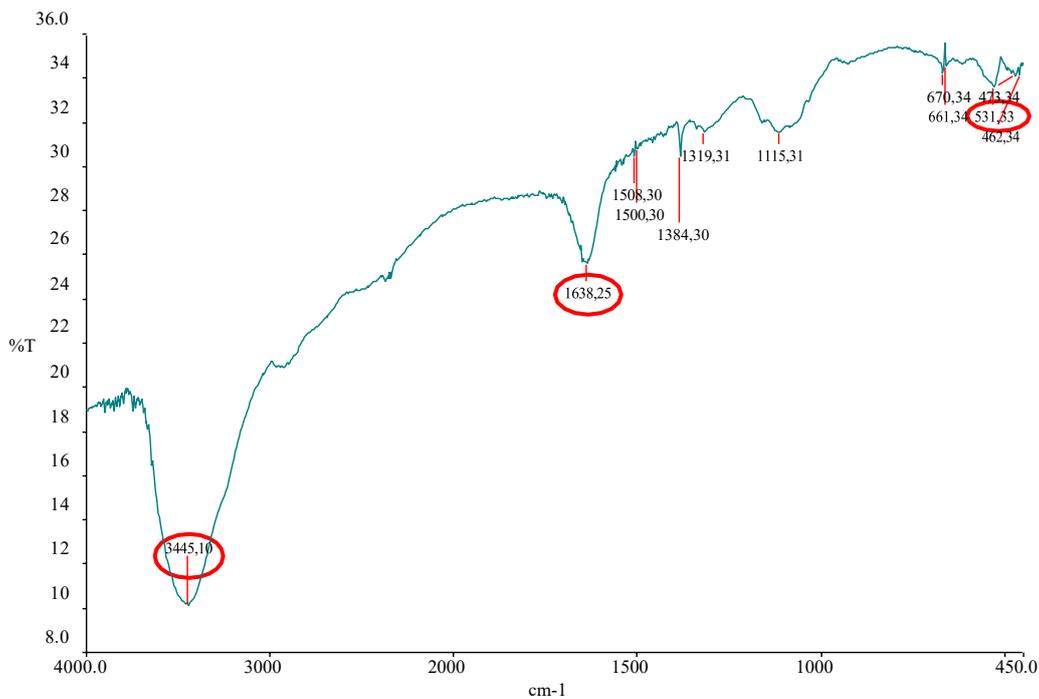


Figure 2. (b) FTIR Spectra of Microbial Cellulose-PVP-HA Scaffold (50:100) (Sample B)

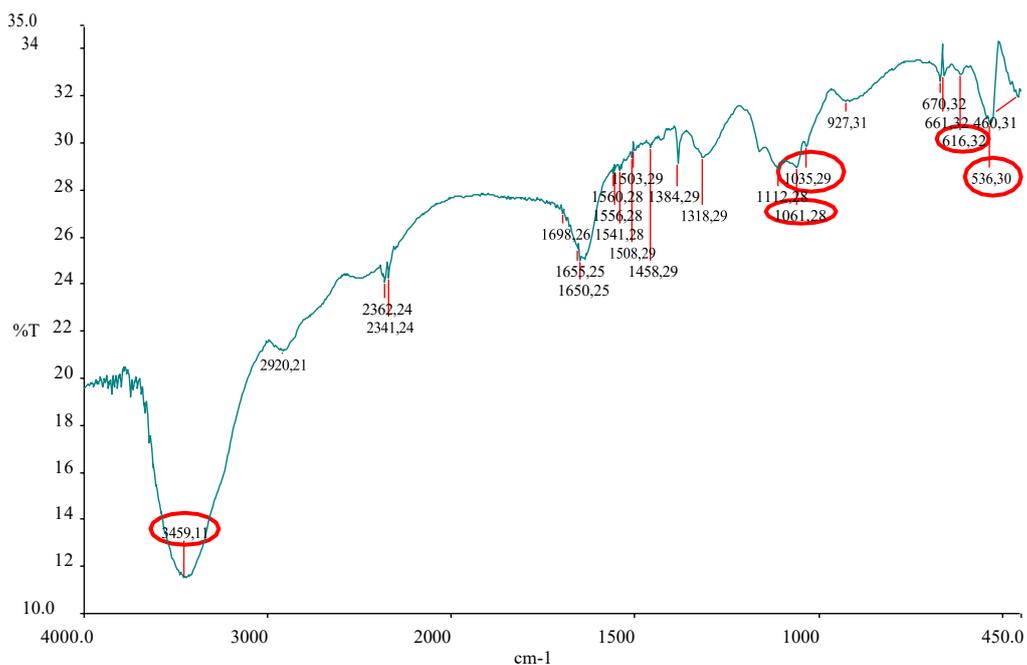


Figure 3. (a) FTIR Microbial Cellulose-HA Scaffold Spectra (75:75) (Control C)

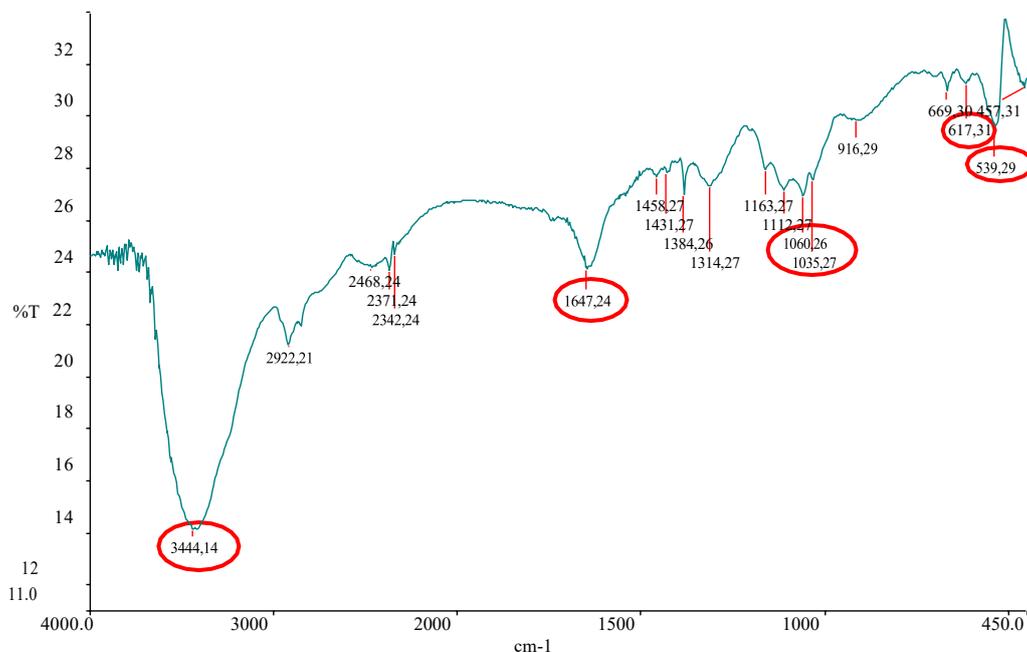


Figure 3. (b) FTIR-Microbial Cellulose-PVP-HA Scaffold Spectra (75:75) (Sample C)

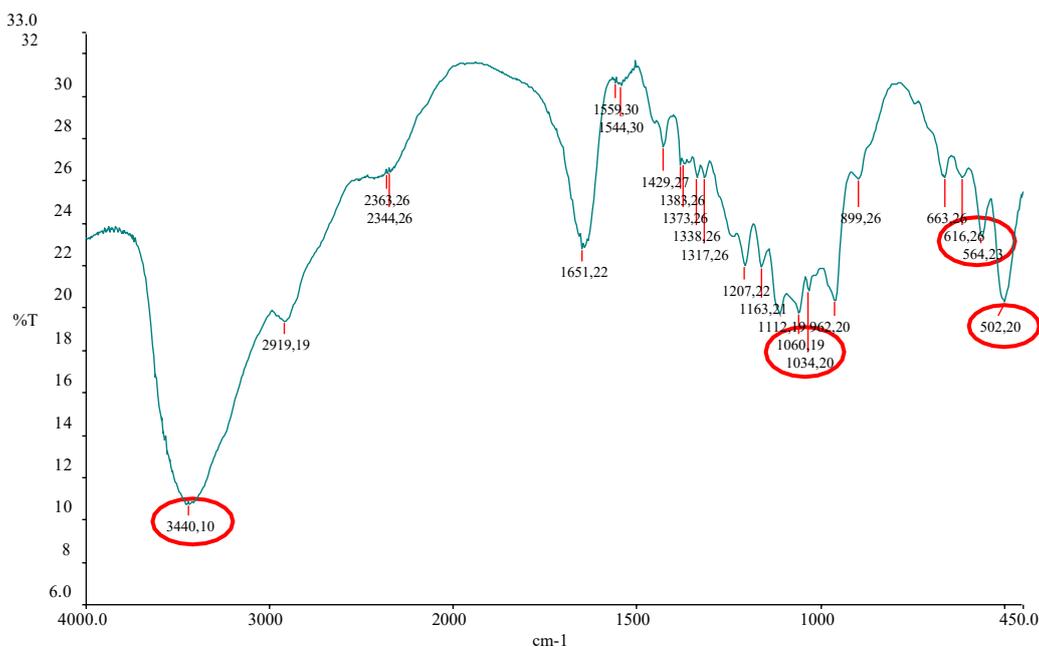


Figure 4. (a) FTIR Spectra of Microbial-HA Cellulose Scaffold (100:50) (Control D)

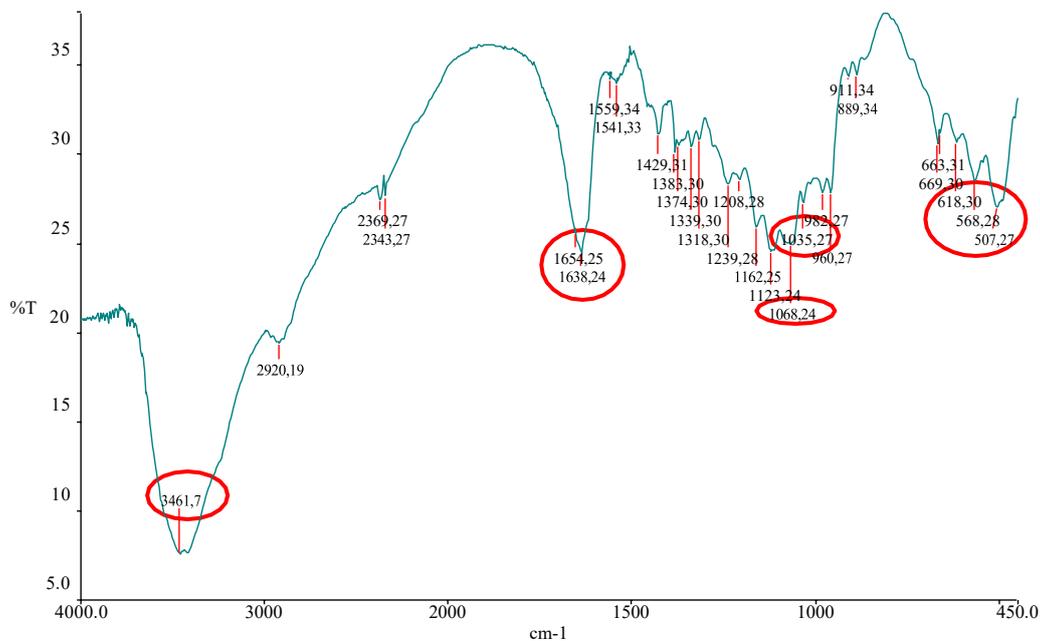


Figure 4. (b) FTIR-Microbial Cellulose-PVP-HA Scaffold Spectra (100: 50) (Sample D)

Figure 1. (a) is the result of FTIR from microbial-hydroxyapatite cellulose scaffold which shows the existence of an identical functional group in accordance with the chemical formula of hydroxyapatite, namely $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Specific functional groups that show apatite crystals of microbial-hydroxyapatite cellulose scaffold samples (in Control A) are PO_4^{3-} groups in wave numbers $1035,32 \text{ cm}^{-1}$, $1060,31 \text{ cm}^{-1}$, $617,23 \text{ cm}^{-1}$ dan $536,37 \text{ cm}^{-1}$ (Petrovic et al., 2012), because the presence of phosphate groups formed indicates that microbial cellulose used is conducive to inducing hydroxyapatite formation (Jordan, 2012). Then there is also the O-H stretching functional group at the wave number $3420,20 \text{ cm}^{-1}$. Then there is the CO_3^{2-} functional group at wave numbers $1458,19 \text{ cm}^{-1}$, $1503,35 \text{ cm}^{-1}$, $1508,34 \text{ cm}^{-1}$, $1542,34 \text{ cm}^{-1}$, dan $1560,34 \text{ cm}^{-1}$ (Sopyan et al., 2002). For identical functional groups that are in accordance with the chemical formula of cellulose that is $[\text{C}_6\text{H}_{10}\text{O}_5]_n$ which is

indicated by the presence of O-H stretching functional groups at wave number $3420,20 \text{ cm}^{-1}$, O-H bending function groups at wave number $1650,31 \text{ cm}^{-1}$ (Saska et al., 2011), C-O functional groups at wave numbers $1035,32 \text{ cm}^{-1}$, $1060,31 \text{ cm}^{-1}$, dan $1112,31 \text{ cm}^{-1}$ (Choirul et al., 2007). According to Saska et al., (2011) the presence of O-H functional groups at wave numbers $\sim 3500 \text{ cm}^{-1}$ indicates the interaction between O-H cellulose groups and O-H hydroxyapatite. FTIR results in Figure 4.1. generally identical to the FTIR results in Figure 3, 5, 7, this is due to the similarity of scaffold compilers namely microbial cellulose with hydroxyapatite, so that the functional groups contained in each scaffold each (a) represents a control ie microbial cellulose-HA) the same, only differing in the value of the wave number. In Figure 1. (b) is the result of FTIR microbial cellulose-PVP-hydroxyapatite scaffold (Sample A), where Poly (Vinyl Pyrrolidone) is added because PVP can effectively join large amounts

of Ca^{2+} ions through strong ion-polar interactions due to the presence of a group of ions C-N and C=O, so that the strongly formed Ca^{2+} ion can facilitate to join PO_4^{3-} and CO_3^{2-} and then increase nucleation in the formation of apatite crystals and minimize mineralization time (Yin et al., 2011). The graph of FTIR results shows that there is an additional formation of C = O functional groups in the wave number $1651,16 \text{ cm}^{-1}$ derived from PVP compounds. Then there is the OH stretching function group in the wave number $3414,11 \text{ cm}^{-1}$, compared to the OH stretching function group in Control A, the wave number changes from $3420,20 \text{ cm}^{-1}$ to $3414,11 \text{ cm}^{-1}$, the wave number in the group decreases O-H indicates the very strong interaction of O-H cellulose groups with C=O groups from PVP. Then obtained the functional groups at wave numbers $1383,18 \text{ cm}^{-1}$ and $1458,19 \text{ cm}^{-1}$ belonging to the heterocyclic group of PVP (Yin et al., 2011). For specific functional groups that indicate the presence of hydroxyapatite content in Sample A is indicated by the presence of PO_4^{3-} groups at wave numbers $1035,15 \text{ cm}^{-1}$, $1060,14 \text{ cm}^{-1}$, $617,23 \text{ cm}^{-1}$, and $536,37 \text{ cm}^{-1}$ (Petrovic et al.,

2012). Also visible is the functional group CO_3^{2-} at wave number $1458,19 \text{ cm}^{-1}$ (Sopyan et al., 2002). FTIR results in Figure 2. generally identical to the FTIR results in Figure 4, 6, 8, this is due to the similarity of scaffold constituents namely microbial cellulose-PVP-hydroxyapatite, so that the functional groups contained in each scaffold ((b) represent the treatment sample (microbial cellulose-PVP-HA)), only differing in the wave number .

From the graph throughout Figure 1. up to Figure 8. it appears that both microbial-hydroxyapatite cellulose scaffold (Controls A, B, C, and D) and microbial-PVP-hydroxyapatite cellulose scaffold (Samples A, B, C, and D) each contain CO_3^{2-} groups - according to Saska et al., (2011) indicates the absorption of CO_2 from the air. The presence of the CO_3^{2-} group also indicates that the hydroxyapatite formed in this microbial cellulose scaffold is a hydroxyapatite containing carbonate. According to Yin et al., (2011) PVP encloses the surface of microbial cellulose so that the formation of hydroxyapatite containing carbonates. The content of functional groups in all samples can be seen in Table 1.

Table 1. Wave Group Function Number of Microbial-HA Cellulose Scaffold and Microbial-Cellulose-PVP-HA

Name sample	O-H	PO ₄ ³⁻	C-O	C=O
Control A	3420,20	1035,32 1060,31 536,37	1112,31 1314,33	1650,31 1655,31
Sample A	3414,11	1035,15 1060,14 536,21	1111,15 1631,16 1315,17	1651,16
Control B	3445,5	1061,22 535,23	1112,22 1163,23 1316,22	1637,18
Sample B	3445,10	531,33	1115,31 1319,31	1638,25
Control C	3459,11	1035,29 1061,28 536,30	1112,28 1318,29	1650,255 1655,25 1698,26
Sample C	3444,14	1035,27 1060,26 539,29	1112,27 1163,27 1314,27	1647,24
Control D	3440,10	1034,20 1060,19 502,20 564,23	1112,19 1163,21 1207,22 1317,26	1651,22
Sample D	3461,7	960,27 982,27 1035,27 1068,24 507,27 568,28	1123,24 1162,25 1239,28 1318,30	1638,24 1654,25

Morphological Test Results and Element Identification

Morphological tests and identification of elements were carried out using Scanning Electron Microscopy-Energy Dispersive X-ray (SEM-EDAX), this was done to determine the shape of the surface morphology of the sample and also identify the elements contained in the sample. The sample to be tested is placed in a holder and then coated with gold and palladium (Au-Pd) samples conductive to electricity flow

(conductive when subjected to electron beam) (Srivastava et al., 2012), then placed in a specimen chamber for subsequent analysis using electron beams. Morphological test results using SEM will later be displayed on a computer screen in the form of an image. The results of SEM tests can show the size of the pore formed, in this case using a magnification of 100-20.000 times.

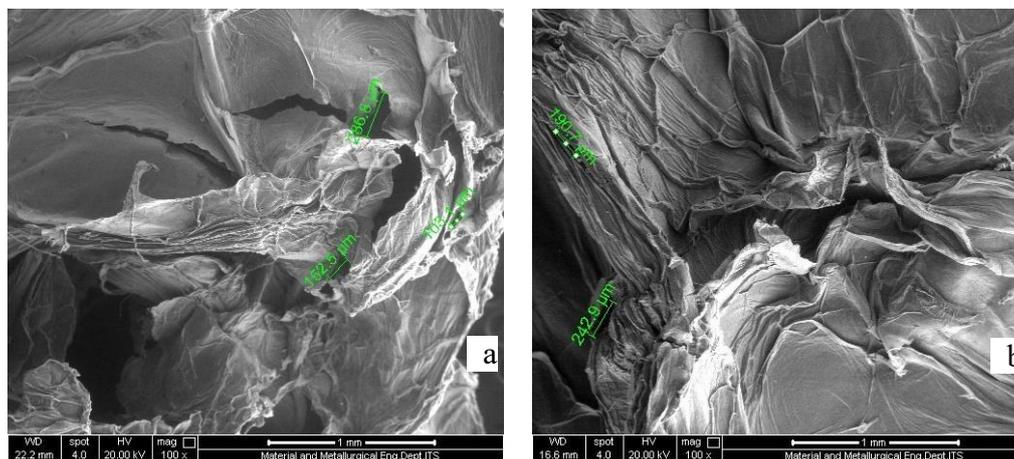


Figure 9. (a) Surface Structure of Microbial-HA Cellulose Scaffold (25: 125) (Control A); (b) Surface Structure of Microbial Cellulose-PVP-HA Scaffold (25: 125) (Sample A)

Figure 9. (a) shows the surface morphology of HA-microbial cellulose scaffold (Control A) where there are pores with sizes between $103.1 \mu\text{m}$ - $186.8 \mu\text{m}$ while in Figure 4.9. (b) belonging to the microbial-PVP-HA cellulose scaffold (Sample A) having pores of about $190.7 \mu\text{m}$ - $242.9 \mu\text{m}$. From the SEM results show that by adding PVP to microbial-hydroxyapatite cellulose scaffold makes the pore size bigger.

In Figure 10. (a) shows the surface morphology of the microbial-PVP-HA (Sample B) microbial cellulose scaffold which has pores of about

$115.2 \mu\text{m}$ - $279.7 \mu\text{m}$, and in Figure 4.10. (b) shows the surface morphology in Sample C where there are pores with a size of about $131.0 \mu\text{m}$ - $291.2 \mu\text{m}$.

SEM results in Figure 9. (a), Figure 4.9. (b), Figure 4.10. (a) and Figure 4.10. (b) each can only be seen in magnification 100 times, 125 times and 150 times. This makes the surface structure of the sample that can be seen only on the outside does not reach the inside, because when viewed in magnification more than about 150 times the resulting image is not clear.

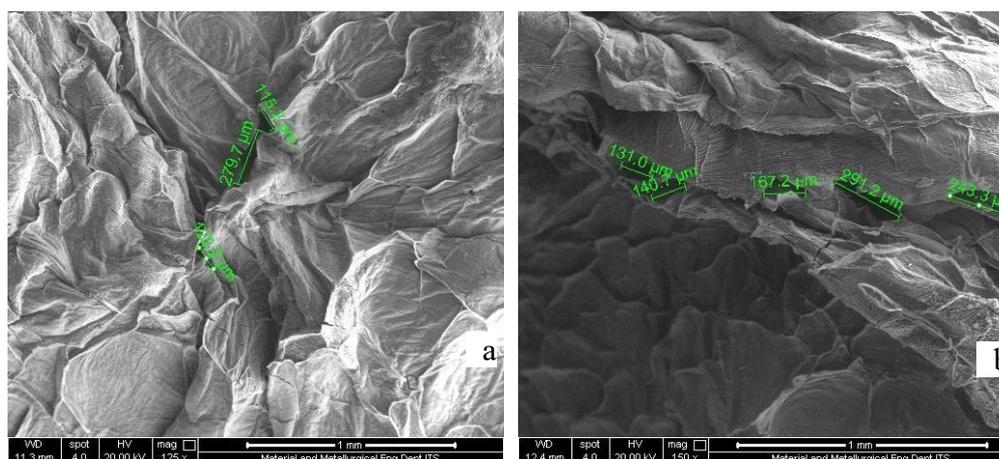


Figure 10. (a) Surface Structure of Microbial Cellulose-PVP-HA Scaffold (50: 100) (Sample B); (b) Surface Structure of Microbial Cellulose-PVP-HA Scaffold (75:75) (Sample C)

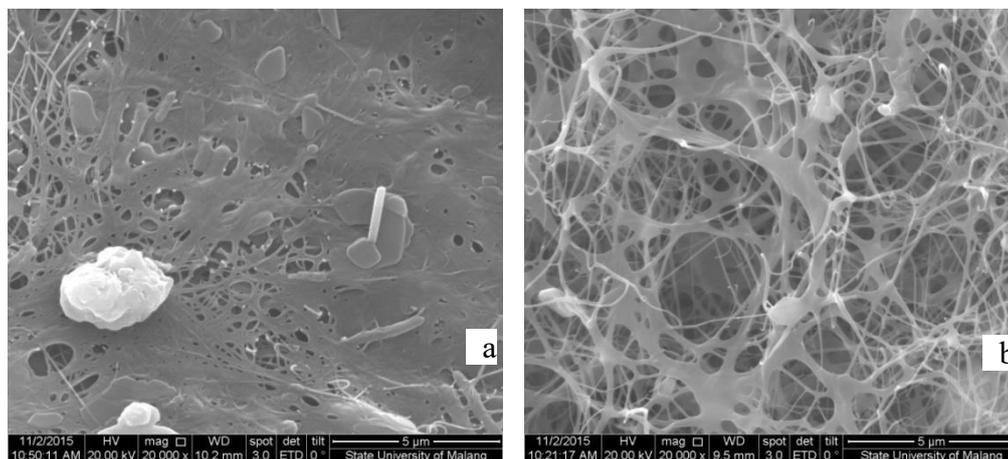


Figure 11. (a) Surface Structure of Microbial-HA Cellulose Scaffold (100: 50) (Control D); (b) Surface Structure of Microbial Cellulose-PVP-HA Scaffold (100: 50) (Sample D)

Figure 11 (a) shows the surface morphology of the microbial-HA cellulose scaffold (Control D) which shows that the resulting pores are still small in comparison to Figure 11 (b) showing the surface morphology of the microbial-PVP-HA cellulose scaffold (Sample D).

All scaffold samples produced using the freeze dried method in the formation of pores, according to Zhu and Chen, (2013) the freeze dried method can only form random pore structures that cause the pore image in SEM to depend on the part where the image was taken, because of the proportion of pores generated using this method cannot be controlled with certainty. There are various pore sizes produced, according to Zhu and Chen, (2013) scaffolds which have a pore size of around $5\ \mu\text{m}$ will be good in the neo-vascularity process, for scaffolds which have a pore size of about $5 - 15\ \mu\text{m}$ will be beneficial for fibroblast growth, scaffolds which have a pore size of around $20 - 125\ \mu\text{m}$ can have an

effect in the regeneration of adult mammalian skin, while scaffolds which have a size of more than $500\ \mu\text{m}$ will be able to play a role in the formation of fibrovascular tissue.

In all samples it was found that the pores formed were around $150-300\ \mu\text{m}$, this is in accordance with the Vaccaro literature, 2002 which states that scaffold with a size of about $150-500\ \mu\text{m}$ is the optimal size for interface activity between biomaterials with the host, bone growth, and implants resorption. Therefore, the pores produced in all samples are eligible to become a scaffold for bone healing applications. However, recent research shows that scaffolds that have both micropore and macropore (pore multiscales) can work better in accelerating bone growth compared to scaffolds that only have macropores (Susmita et al., 2012). Micropores are pores smaller than $10\ \mu\text{m}$ while macropores are pores with a size of more than $50\ \mu\text{m}$ (Miao et al., 2010).

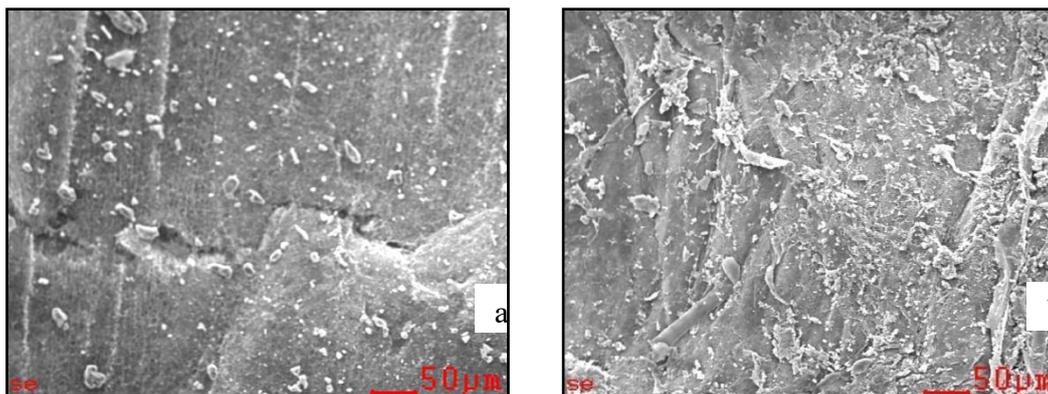


Figure 12 (a) Surface Structure of Microbial-HA Cellulose Scaffold (Control); (b) Surface Structure of Microbial Cellulose-PVP-HA Scaffold

Figure 12. (a) and (b) show the scaffold morphological structure that describes the spread of apatite crystal formation. Where in Figs 12 (a) which is the control of formation of apatite crystals is not solid when compared to Figure 12 (b) which is a microbial-PVP-HA cellulose scaffold. This indicates that PVP makes the formation of more apatite crystals so that it becomes solid. This is because the addition of PVP to microbial-hydroxyapatite cellulose scaffold is considered to make the surface of microbial cellulose become active so that it can increase the nucleation rate of apatite crystals and induce the formation of hydroxyapatite crystals (Yin et al., 2011).

After the SEM test to determine the surface morphology of the scaffold, an EDAX test was

performed to identify the constituent elements of the scaffold. The elements identified in this case are Ca and P elements to obtain Ca/P ratio values, because in this research synthesis of microbial-hydroxyapatite cellulose is synthesized, in which hydroxyapatite is obtained by immersion of microbial cellulose into CaCl_2 and KH_2PO_4 solutions, where variations in ion concentration are carried out. Ca^{2+} and PO_4^{3-} ions, and a comparison between microbial-hydroxyapatite cellulose without the addition of PVP and microbial-hydroxyapatite cellulose added by PVP. For this reason, this test is carried out in order to be able to show the Ca/P ratio in the resulting scaffold. The test results are shown in Table 2.

Table 2 EDAX Results of Ca / P Microbial-HA Cellulose Scaffold Ratio

Name Sample	Ca	P	Rasio Ca/P
Control A	0,13	12,39	0,010492
Sample A	0,42	4,06	0,103448
Control B	0,18	14,11	0,012757
Sample C	0,83	10,39	0,079885
Control D	2,16	4,47	0,483221
Sample D	1,85	3,06	0,604575

The EDAX results showed that the highest Ca / P ratio was obtained by microbial-PVP-

hydroxyapatite cellulose scaffold with variations in $\text{Ca}^{2+} : \text{PO}_4^{3-}$ ion concentration of

100 mM: 50 mM which was 0.6046, and the lowest Ca/P ratio was obtained by microbial scaffold -hydroxyapatite with variations in the concentration of Ca^{2+} : PO_4^{3-} ions of 25 mM: 125 mM which is equal to 0,0104.

From the results it was also found that the samples added to PVP (samples A, B, C, D) when compared to samples without the addition of PVP (control A, B, C, D) had a higher Ca / P ratio, evidenced in the ratio of Ca / P sample A is 0,103448 and Ca / P ratio of control A is 0,010492, which proves an increase of 10x between control scaffold compared to treatment scaffold, also in sample D is 0,604575 and control D is 0,483221 which proves the occurrence 1.25x increase between control scaffolds versus treatment scaffolds. This is due to the addition of PVP which is a bioactive polymer that makes the surface of microbial cellulose become active because PVP encloses the entire surface of microbial cellulose fibers so that the ability of the nucleating rate of apatite microbial cellulose can be increased so that it stimulates the formation of hydroxyapatite crystals, because PVP can effectively bind Ca^{2+} ions in greater amounts through strong ion-polar interactions because of the C-N and C=O groups contained in PVP so as to facilitate the fusion of Ca^{2+} with PO_4^{3-} and CO_3^{2-} (Yin et al., 2011).

The best Ca / P ratio was obtained in sample D where the Ca / P ratio produced was 0.6046. However, all samples have Ca / P ratio values less than 1.67, this is due to many factors that affect Ca / P ratio values, especially in synthesis procedures, such as precursor reagents, impurity content, morphology and crystal size, concentration (mainly due to high PO_4^{3-} ion

concentration compared to Ca^{2+} ion concentration, pH, temperature, and temperature treatment given during drying or sintering (Eslami et al., 2008). Although the resulting scaffold has a Ca / P ratio of less than 1.67, the scaffold can still be a candidate for bone healing, because according to Yin et al., (2011) when the Ca / P ratio is lower than the standard value, which is 1, 67, still making scaffold can be applied for bone healing, because a Ca / P ratio below 1.67 is more useful because it allows for fast bonding with bone hosts. The low Ca / P ratio is also beneficial because it accelerates the dissolution of Ca^{2+} ions, which is accompanied by an increase in local pH at the scaffold interface with the tissue causing the creation of an ideal pH for alkaline phosphatase activity so that osteoblast proliferation and bone matrix synthesis are increased (Saska et al., 2011).

Degradation Test Results

A biomaterial must have a variety of properties, one of which is biodegradable. Biodegradable is the ability of a biomaterial to degrade at a certain time when used in the body. A scaffold is needed to have biodegradable properties because this material is expected to be completely degraded in the body after new tissue (bone) has formed. To test the biodegradable properties of microbial-HA cellulose scaffold, degradation test was carried out using a medium called Simulated Body Fluid (SBF) which has a pH of 7.27. The scaffold was immersed in SBF for 21 days and then evaluated by weight of the scaffold after being immersed. The results of the microbial-

hydroxyapatite cellulose scaffold degradation

test can be seen in Table 3

Table 3 Results of Microbial-HA Cellulose Scaffold Degradation Test

Name Sample	W ₀ (gram)	W ₁ (gram)			Laju Degradasi (%)		
		Week I	Week II	Week III	Week I	Week II	Week III
Control A	0,0254	0,0164	0,016	0,0152	35,43307	37,0079	40,1575
Sample A	0,0194	0,0129	0,0124	0,0123	33,50515	36,0825	36,5979
Control B	0,0204	0,0131	0,0129	0,0125	35,78431	36,76471	38,7254
Sample B	0,0218	0,0145	0,0132	0,0129	33,48624	39,44954	40,8256
Control I C	0,0177	0,0139	0,0122	0,0109	21,46893	31,0734	38,4181
Sample C	0,0153	0,0121	0,0115	0,0113	20,91503	24,8366	26,1438
Control D	0,0242	0,0218	0,0187	0,0162	16,1157	22,7273	33,0579
Sample D	0,0231	0,0199	0,0186	0,0179	13,85281	19,4805	22,5108

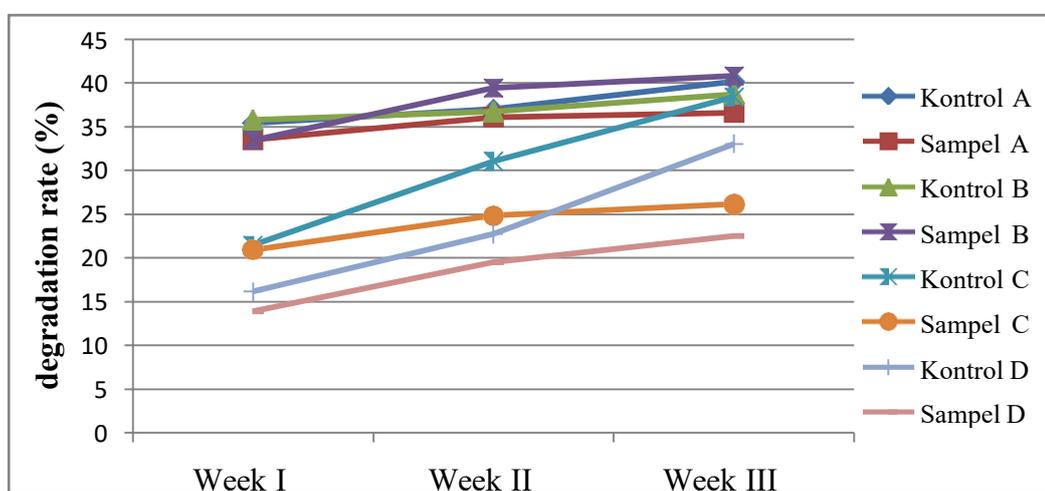


Figure 13. Graph of Results of Microbial-HA Cellulose Scaffold Degradation Test

From the results of the degradation test in Table 3 obtained graphs like in Figure 13 which illustrates the decrease in mass in each sample in week I, week II, and week III. This decrease in mass indicates the degradation of the sample, in general the rate of degradation of the entire sample for 21 days has increased. The best results obtained microbial-PVP-hydroxyapatite scaffold with variations in $\text{Ca}^{2+} : \text{PO}_4^{3-}$ ion concentrations of 100 mM : 50 mM ie in week I of 13.8528%, week II of 19.4805%, and week III of 22.5108%, where the increase in degradation rate of this sample is not too

significant so it is considered more stable degradation rate.

Overall scaffold which has more Ca / P ratio has a lower degradation rate, this is because the high Ca/P ratio indicates that more hydroxyapatite is produced so that it makes a low degradation rate because hydroxyapatite has a low degradation rate (Kusnetsova et al ., 2014) so it is possible that the first degradation is cellulose in microbial cellulose because microbial cellulose is easily degraded in SBF solution (Darwis, 2012). Therefore, scaffold can be applied as a bone healing candidate.

Porosity Test Results

The porosity test is carried out to find out how much porous content is formed in the scaffold. This test is carried out using the liquid displacement method, where the liquid used is ethanol. The use of ethanol because ethanol will be absorbed into the scaffold without changing

the shape of the scaffold (development or shrinking) (Wassanai et al., 2014). The test is done by immersing the scaffold into ethanol for 48 hours, then the weight is calculated and calculated% Porosity using the equation 2.1. The porosity test results for all samples are shown in Table 4

Table 4 Results of Microbial-HA Cellulose Scaffold Porosity Test

Name Sample	V ₁ (gram)	V ₂ (gram)	V ₃ (gram)	% Porositas
Control A	0,0055	3,3033	1,4642	79,32
Sample A	0,0093	2,9596	1,4348	93,49
Control B	0,0067	3,4206	1,4912	76,94
Sample B	0,0078	3,4595	1,1749	51,09
Control C	0,0082	3,2141	1,4871	85,63
Sample C	0,0061	3,3066	1,542	87,04
Control D	0,0492	4,2895	1,9918	84,55
Sample D	0,0258	3,8587	1,8243	88,40

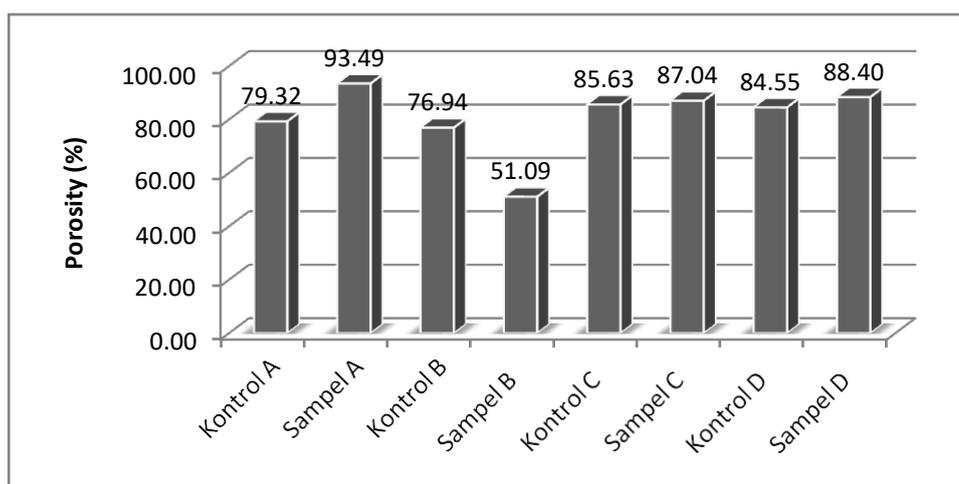


Figure 14 Graph of Microbial-HA Cellulose Scaffold Porosity Test Results

From the porosity test results in Table 4.4. graphs obtained as in Figure 14 illustrates the porosity percentage content in each sample, where each sample has a different porosity content. The highest porosity percentage results were obtained by microbial-PVP-hydroxyapatite cellulose scaffold with variations in Ca²⁺ ion concentration by 25 mM: 125 mM (sample A) that is 93.49%. The

smallest porosity percentage was obtained by microbial-PVP-hydroxyapatite cellulose scaffold with variations of Ca²⁺: PO₄³⁻ ion concentration of 50 mM: 100 mM (sample B), which amounted to 51.09%. The high percentage of porosity value is caused by the use of the freeze dried method, according to Al-Shamary, (2013) scaffolds that use the freeze dried method in forming pores and drying can

cause porosity of ~ 88%. Scaffolds which have high porosity can facilitate the diffusion of nutrients and metabolic waste and are also very beneficial for cell migration and neo-vascularity (Zhu and Chen, 2013).

The highest porosity result was obtained in sample A which was 93.49%, but the best results were obtained from microbial-PVP-hydroxyapatite cellulose scaffold with variations in $\text{Ca}^{2+} : \text{PO}_4^{3-}$ ion concentration of 100 mM: 50 mM, because porosity exceeding 90% would reduce the strength mechanical and affect the structural integrity of the scaffold before replacing new bone (Karageorgiou et al., 2005).

From the entire results of the bacterialization, the best results were obtained on the microbial-PVP-hydroxyapatite cellulose scaffold with variations in $\text{Ca}^{2+} : \text{PO}_4^{3-}$ ion concentration of 100 mM: 50 mM, because through the FTIR test it was proved that there were PO_4^{3-} and CO_3^{2-}

CONCLUSION

1. The effect of variations in Ca^{2+} and PO_4^{3-} concentration with the addition of Poly (Vynil Pyrrolidone) (PVP) to microbial-hydroxyapatite cellulose scaffold is to increase the formation of apatite crystals because according to test results using SEM-EDAX shows that the Ca/P ratio in the scaffold has increased this due to Ca^{2+} ion concentration which is greater than PO_4^{3-} ion concentration - making Ca/P ratio becomes larger, because Ca^{2+} will bind PO_4^{3-} causing more calcium phosphate to be formed so that the hydroxyapatite produced will be more,

compounds, where this compound was part From the hydroxyapatite compound, the highest Ca / P ratio was also obtained, which was 0.6046, with an average degradation rate of 18.617%, and porosity percentage of 88.4%. According to Wang et al., (2011) porosity and pore size of a scaffold have an important role in bone formation in vitro and in vivo. The more pores in the structure of the scaffold, the scaffold has a good condition for cell growth, cell adhesion, cell proliferation, and transportation of nutrients and metabolic waste. The large percentage of porosity and pore size will increase bone growth and osteointegration of the implant after it is implanted in the body (Karageorgiou et al., 2005). Therefore, microbial cellulose-PVP-hydroxyapatite with variations in $\text{Ca}^{2+} : \text{PO}_4^{3-}$ ion concentration of 100 mM: 50 mM can be a scaffold candidate for bone healing.

because calcium phosphate is a constituent of hydroxyapatite.

2. The best results are obtained from microbial-PVP-hydroxyapatite cellulose scaffold with variations in Ca^{2+} ion concentration: PO_4^{3-} of 100 mM : 50 mM, because through the FTIR test it was proven that there are PO_4^{3-} compounds - and CO_3^{2-} where these compounds are part of the hydroxyapatite compound, also the highest Ca / P ratio is 0.6046, with an average degradation rate of 18.617%, and porosity percentage of 88.4%.

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