Full Paper

FABRICATION AND CHARACTERIZATIONS OF GELATIN/CHITOSAN WITH ALOE VERA AND ACHATINA FULICA SP MUCUS AS SCAFFOLD FOR SKIN TISSUE ENGINEERING

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Abstract

Scaffold is a biomaterial widely used in tissue engineering. Scaffold is temporary porous structure which contain extracellular matrix. It serves as scaffolding which is required for cells infiltration and physical support to guide the cell proliferation and differentiation into the targeted functional tissues. Scaffold must be biocompatible, small pore size, flexible and support regenerative application. Aloe vera as natural resource, it has capability in accelerating wound healing, facilitating the inflammation, increasing wound contraction and epithelialization, and increasing organization of the regenerated tissue. Snail mucus (SM) has capability in inhibiting bacterial growth. This study aims to synthesize and characterize a scaffold made of gelatin-chitosan-Aloe vera (AV) - Achatina fulica sp mucus. The method is to synthesize scaffold with its compositions, gelatin-chitosan (1: 1 ratio) which is dissolved into 0.05 M acetic acid, then a variation of Aloe vera (AV) and snail mucus (SM) at 0% AV; 0% SM; 0.07 AV; 0.07 SM; 0.15 AV; 0.15 SM; 0.07 AV; 0.15 SM; 0.15 AV; 0.007 SM were mixed with the chitosan-gelatin solution, then used freeze dry method to obtain porous scaffold. Characterization which performed in this research including Fourier-transform infrared spectroscopy (FTIR), Scanning Electron Microscopy (SEM), porosity test, tensile strength test, swelling test, and degradation test. Based on FTIR test, Aloe vera and snail mucus interacted with free amino and hydroxyl groups of chitosan and gelatin, characterized by absorption bands at 2937.59 cm-1 wave numbers which are symmetrical and asymmetrical stretching of (-CH) .SEM test results obtained pore size of 70 - 235 μm. Porosity test results showed that five scaffolds have porosity value of 87-96%; thus, allowing the process of cell proliferation to occur well. The result of physical characteristic test yielded tensile strength of 1.425 MPa on gelatin-chitosan as control sample and 0.732 MPa for sample with 0.15% AV and 0.15% SM. Swelling test showed a variation of scaffold composition with Aloe vera with Achatina fulica sp's mucus having a swelling percentage of 200-520%. The degradation test results showed that the whole sample was not depleted for 21 days; thus, giving time for cell regeneration. Sample with 0.15% Aloe vera and 0.07% snail mucus has some potentials as scaffolds for skin tissue in case of burns wound, due to its morfology, porosity, and degradation.

Keywords: Scaffold, gelatin, chitosan, Aloe vera, snail mucus, burns

1.0 INTRODUCTION

High prevalence level of skin loss due to burn cases needs some regeneration process to return the skin's main function, namely protect the body, organize some physiology features like temperature, and function as sensory system [1]. World Health Organization (WHO) also notes that burns are a global public health problem, accounting for an estimated 180 000 deaths annually [2]. For third degree burns, the wound is too deep and damages organs under the skin, such as muscles, nerves,

bones; moreover, it could not recover spontaneously due to the proliferation of its epithelial layer (re-epithelialization) at the edge of the wound does not occur [3].

The most effective third degree burns healing process requires the role of a scaffold as an important factor in tissue engineering. Scaffold is a temporary porous buffer material in which there is an extracellular matrix functioning as a scaffold for infiltration and physical support to guide cell differentiation and proliferation into targeted functional tissues [4].

Biocomposite of these material is potential to be developed as scaffold for tissue engineering in burn cases, particularly gelatin which has several commercially available properties at low cost, biodegradable, and biocompatibility [5], as well as chitosan which has been widely applied in various biomedical applications, such as tissue engineering and regenerative drugs due to their non-toxic and biocompatible properties [6]. Adding *Aloe vera* can hydrate skin, as it contains anti-inflammatory material [7], while adding *Achatina fulica sp*'s mucus with its anti-bacterial factor can work by attacking or inhibiting the formation of bacterial strains' common parts, such as peptidoglycan layers and cytoplasmic membranes [8].

The ideal scaffold for tissue engineering applications should be biocompatible, has large surface ratios, large volumes, small pore size to control degradation, flexible in mechanical properties, and biologically safe and support medical applications due to organ replacement in order to support regenerative treatment [9]. Suitable micro-scaffold structures, such as average pore size of 63-150 µm and porosity values above 90% [10] has some appropriate mechanical properties, including elongation and tensile strength [11, 12]. The characterizations used include Fourier Transform Infra Red Spectroscopy (FTIR) test, Scanning Electron Microscope (SEM) test, porosity test, tensile strength test, swelling test, and degradation test. Previous research on scaffold synthesis based on gelatine and chitosan was performed by Kakkar at 2014 [13]. In his study,the scaffold mechanical value is less elastic. It is not applicable for skin substitute , because it needs appropriate tensile strength, flexibility and elasticity. In this research, he added Aloe vera and snail mucus to create anti-inflammatory and pain relief scaffold, and glycerol for better flexibility and elasticity.

2.0 METHODOLOGY

Materials

Gelatin, Phosphate Buffer Saline (PBS), acetic acid, NaOH solution, glycerol, Ethanol, $NaBH_4$ solution from UD.SIP (Surabaya, Indonesia), chitosan (DD = 85%) from Sigma Aldrich, snail mucus (*Achatina fulica sp*'s mucus), *Aloe vera* extract (*Aloe vera*),

glutaraldehyde from PT. Kristalindo (Surabaya, Indonesia). Magnetic bar, magnetic stirrer, incubator, freeze dryer, FTIR test tool (8400 Shimadzu), SEM test tool (FEI, Inspect S50 Jepang), and Tensile Strength test equipment (Autograph Imada HV-500 NII).

This study used 3 variables, which are control variables (2% gelatin and 1% Chitosan), independent variables (*Aloe vera* concentration and snail mucus at ratio (0.07: 0.07; 0.15: 0.15; 0.07: 0.15; 0.15: 0.07 w/ v), and dependent variables (gelatin/chitosan-based biocomposites characteristic with *Aloe vera* extract and snail mucus as the scaffolds).

Methods

Preparing gelatin-chitosan-*Aloe vera*-snail mucus scaffold is initially conducted by mixing 2% gelatin solution and 1% Chitosan solution with 1: 1 ratio using magnetic stirrer at 500°C for 2 hours. The incorporation of Aloe vera and snail mucus into scaffolds based on gelatin and chitosan is tend to alter the structure, composition and biodegradability property, as good as the potential effect on bioactivity. Aloe vera (A) and snail mucus (S) blended Gelatine (G)-Chitosan (Ch) composite was formulated by mixing different weighted quantities of A and S with the G-CH solution to obtain a final concentration *Aloe vera* and snail mucus extracts with various concentrations 0:0 (Sample A), 0.07: 0.07 (Sample B), 0.15: 0.15 (Sample C), 0.07: 0.15 (Sample D), 0.15: 0.07 (Sample E) (w/ v) are added along with 0.3% Glycerol. After homogeneously mixing it, the authors leave it over for 2 hours inside liquid nitrogen chamber at -190^0 C. Then,it is frozen and dried at -580°C for 18 hours and placed in a desiccator with 10% gluteraldehyde in 90% ethanol. After that, the sample is immersed in 1% NaOH solution, washed twice with distilled water, and dipped in 5% (w/v) $NaBH4$. Finally, the scaffold was frozen at -190°C and frozen-dried for 18 hours. Sample A with no addition of *Aloe vera* and Snail mucus, Sampel B - E with addition of *Aloe vera* and snail mucus concentration (0.07: 0.07; 0.15: 0.15; 0.07: 0.15; 0.15: 0.07 w/v) subsequently are finally characterized. The size of sample for FTIR test, SEM, degradation test, porosity test, swelling test are 1x1 cm in sheet form, for tensile strength 5x1 cm in sheet form. Furthermore, the characterizations for FTIR test, Scanning Electron Microscope (SEM) test, porosity test, tensile strength test, swelling test, and degradation test are performed. There was 3 times repetition for this experiment.

3.0 RESULT AND DISCUSSION

Functional Groups Result

The aliphatic group ($-CH_2$ and $-CH_3$) can be seen in the absorption of wave number 2939.52 cm^{-1} in sample A, 2937.59 cm^{-1} in sample B, 2937.58 cm^{-1}

in sample C, 2937.59 cm^{-1} in sample D, and 2939.52 cm^{-1} in the sample E. At absorption peak of wave number 1658.78 cm^{-1} in sample A, 1658.78 cm^{-1} in sample B, 1653 cm^{-1} in sample C, 1658.78 cm^{-1} on sample D, and 1664.57 cm^{-1} on sample E has a C = O stretching functional group. At absorption of wave number of 1546.91 cm^{-1} in sample A, 1544.98 cm^{-1} in sample B, 1546.91cm^{-1} in sample C, 1544.98 cm^{-1} in sample D, and 1543.05 cm^{-1} on sample E is -NH$_2$ bending. The absorption of wave number 1154.44 cm^{-1} in sample A, 1145.72 cm^{-1} in sample B, 1161.14 cm^{-1} in sample C, 1161.14 cm^{-1} in sample D, and 1130.29 cm^{-1} in the sample E is -COC-glycosidic relationship between chitosan monomers. The presence of C-O stretching groups from the primary alcohol group is shown in the absorption of wave number 1058.92 cm^{-1} in sample A, 1056.99 cm^{-1} in sample B, 1072.42 cm^{-1} in sample C, 1076.28 cm^{-1} in sample D, and 1031.92 cm^{-1} in sample E. The typical peak of gelatin absorption is shown in the absorption of wave numbers 3392.79 cm^{-1} in sample A, 3346.5 cm^{-1} in sample B, 3429.43 cm^{-1} in sample C, 3433.29 cm^{-1} in sample D, and 3346.5 cm^{-1} in sample E, which is a group of hydroxyl groups (-OH) (as seen in Figure 1). The peak of the amine group for pure chitosan in the study conducted by Ahmad, *et al.*, 2011 was 1643 and 1584 cm^{-1} then shifted to 1658.78 and 1546.91 cm^{-1}

in the gelatin / chitosan composite in sample A; 1664.57 cm^{-1} and 1543.05 cm^{-1} in sample B; 1653 cm^{-1} and 1546.91 cm^{-1} in sample C; 1658.78 cm^{-1} and 1544.98 cm^{-1} in sample D; and 1664.57 cm^{-1} and 1543.05 cm^{-1} in sample E in accordance with the vibration of amine bonds in chitosan. Then the peak of the amino group and hydroxyl group becomes wider and deeper indicating the possibility of *Aloe vera* and snail mucus which is interacted with free amino and hydroxyl groups of Chitosan and Gelatin, this is indicated by the absorption band at wave number 2937.59 cm^{-1} in sample B, 2937.58 cm^{-1} in sample C, 2937.58 cm^{-1} in sample D; and 2939,52 cm^{-1} in sample E which is a symmetrical and asymmetrical C-H stretching from (-CH) which marks the presence of a Piranose Ring. There are also wave number absorption at 1238.30 cm^{-1} in sample B, 1249.87 cm^{-1} in sample C, 1249.87 cm^{-1} in sample D, and 1253.73 cm^{-1} in sample E which is a CO stretching group of Ester and Phenol groups so that the spectrum of the sample with the addition of Aloe vera and Snail mucus extract becomes sharper, and there are also absorption of wave numbers at 1031.92 cm^{-1} in sample B, 1031.92 cm^{-1} in sample C, 1037.7 cm^{-1} in sample D, and 1031.92 cm^{-1} in sample E which are C-O stretching from polysaccharides in Aloe vera and Snail mucus [14].
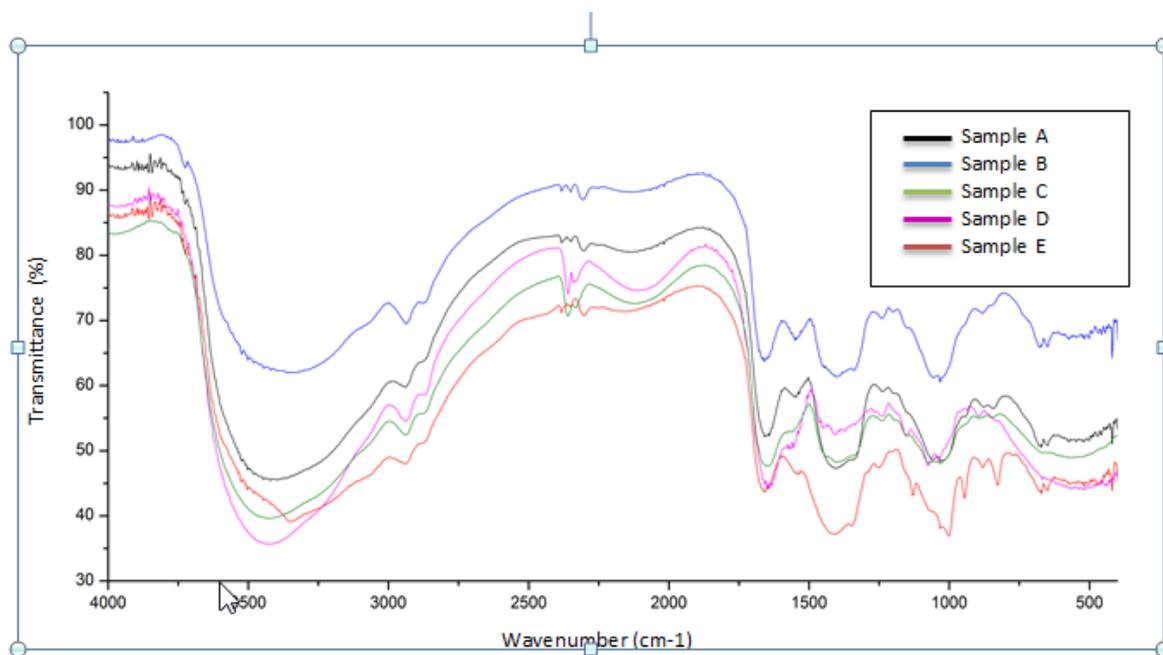

Figure 1 FTIR Spectra of Samples A (0%AV, 0% SM), B (0,07%AV, 0,07% SM), C(0,15%AV, 0,15% SM), D(0,07%AV, 0,15% SM), E (0,15% AV, 0,07% SM)

The peak absorption of NH stretching group which marks the presence of Amida A group, is not seen in this infrared spectra due to the overlap of absorption spectra with OH stretching groups. At wave number absorption 1658.78 cm^{-1} in

sample A, 1658.78 cm^{-1} in sample B, 1653 cm^{-1} in sample C, 1658.78 cm^{-1} in sample D, and 1664.57 cm^{-1} in sample E there is Amida I. The presence of Amida II is indicated by the absorption of wave numbers 1546.91 cm^{-1} in sample A, 1544.98 cm^{-1} in

sample B, 1546.91 cm^{-1} in sample C, 1544.98 cm^{-1} in sample D, and 1543.05 cm^{-1} in sample E. The Cross link of C = O group of gelatin with NH group of chitosan indicates the interaction between Chitosan and Gelatin, thus increasing the intensity of NH bending group at a peak of 3392.79 cm^{-1} in sample A, 3346.5 cm^{-1} in sample B, 3429.43 cm^{-1} in sample C, 3433.29 cm^{-1} in sample D, and 3346.5 cm^{-1} in sample E. The peak of the amino group and hydroxyl group becomes wider and deeper indicates the possibility of *Aloe vera* and snail mucus are interacted with free amino and hydroxyl groups from chitosan and gelatin only in samples B, C, D, and E, indicated by the absorption band at wave numbers 2937.59 cm^{-1} in sample B, 2937.58 cm^{-1} in the sample C, 2937.59 cm^{-1} in sample D, and 2939.52 cm^{-1} in sample E, which is a symmetrical and asymmetrical C-H stretching from (-CH) which marks the presence of a Pyranose Ring. Then there is the absorption of wave number 1238.20 cm^{-1} in sample B, 1249.87 cm^{-1} in sample C, 1249.87 cm^{-1} in sample D, and 1253.73 cm^{-1} in sample E which is a CO stretching group of Ester and Phenol groups so that the

spectrum in the sample with an addition *Aloe vera* and snail mucus extracts become sharper. There is also the absorption of wave numbers at 1031.92 cm^{-1} in the sampleB, 1031.92 cm^{-1} in samples C, 1037.70 cm^{-1} in sample D, and 1031.92 cm^{-1} in sample E, which is a C-O stretching of polysaccharides in *Aloe vera* and Snail mucus [14]. Based on the functional group test, Chitosan / Gelatin scaffold with variation of *Aloe vera* and snail mucus is showed the occurrence of Gelatin and Chitosan binding. This can be proven based on the mixture of absorption characteristics of amine group of chitosan and carboxylic acid group of gelatin.

Morphological Analysis

SEM test aims for morphology test to examine surface structure, pore diameter, porous layer, non-porous layer, and membrane thickness [15]. The samples were placed on a holder and inserted into a recorder tool to be subsequently displayed on computer screen, as shown in Figure 2.
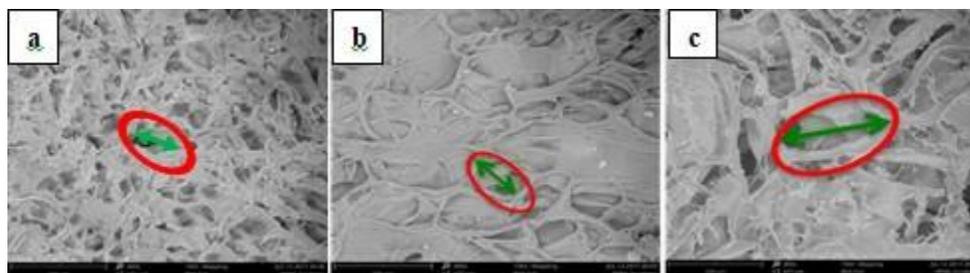

Figure 2 (a) SEM result of sample with concentration of Aloe vera and snail mucus 0.15: 0.07 w/v (sample E)(b) SEM result of sample with concentration of Aloe vera and snail mucus (sample A) (A) 0 : 0 w/v (control). (c) SEM result of sample with concentration of Aloe vera and snail mucus 0.07: 0.07 w/v (sample B)

The sample used in the test were the sample with various concentration of *Aloe vera* and snail mucus, which are sample sample A (0 : 0 w/v), sample B (0,07 : 0,07 w/v) and sample E (0.15: 0.07 w/v). The calculation of pore size in SEM using measurement from the scale bar (reference length). SEM test results of the scaffold surface pore sizes recorded an average value of 117.3 $\pm$ 1,8 μm for sample A, 206.3 $\pm$ 1,7 μm for sample B and 78.5 $\pm$ 1,03 μm for sample E. The best sample with various concentration of *Aloe vera* and snail mucus is 0.15: 0.07 w/v. As shown in Figure 2 sample E has smaller pore sizes than sample A (control) and sample B. The pore sizes used in scaffold applications were 63 - 150μm [10], the pores were in the range for scaffold applications,which is better if it is compared to the previous research by Angulo and Sobral [14] with the pore size 93-296 μm. The addition of *Aloe vera* and snail mucus increases average pore size and porosity and causes some changes in pore architecture [16]. However, the decrease in pore size as the concentration of *Aloe*

vera increases in sample E can be caused by the *Aloe vera* content with macromolecules into the mixed system [23].

Porosity Analysis

Porosity test aims to determine the level of scaffold porosity using fluid transfer method at 25°C with Pycnometer. Absolute ethanol is used as a displaced fluid, because it could easily penetrate the scaffold and not trigger either shrinking or swelling. The porosity value was obtained through weight calculation using the predetermined equation. Analyses of porosity, were performed in triplicate/three times. Based on Figure 3, the control sample recorded porosity value of 80% and the porosity of scaffold with *Aloe vera* and snail mucus recorded porosity ranges of 87-96%. While the data of porosity of each sample are sample A 80% $\pm$ 1, sample B 96% $\pm$ 1, sample C 89 $\pm$ 1, sample D 87% $\pm$ 1,5, sample E 94% $\pm$ 1. The condition of porosity result

are corresponded to a fairly high porosity range which possibly to facilitate cell proliferation .The outer thick layer of *Aloe vera*, or rind, comprised of 15–20 cells, has a protective function and synthesizes carbohydrates and proteins.Vascular bundles inside the rind are composed of xylem and phloem [2]. Xylem helps to transport water and minerals from roots to leaves and phloem helps to transport starch and other small organic molecules [23]. AV and snail mucus have shown excellent porosity and biological properties.Polysaccharides which are provided by *Aloe vera* and snail mucus can improve the material porosity, by allowing entry for water [14].
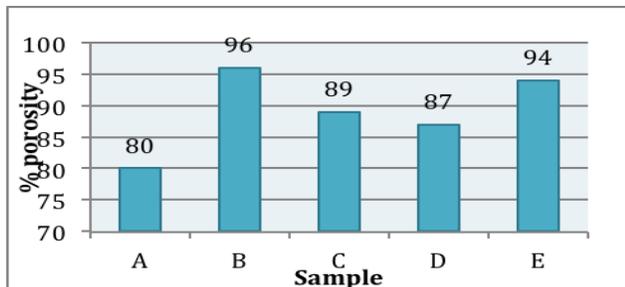

Figure 3 (a) Porosity test results of sample A (0%AV, 0% SM), B (0,07%AV, 0,07% SM), C(0,15%AV, 0,15% SM), D(0,07%AV, 0,15% SM), E (0,15% AV, 0,07% SM)

Tensile Strength Test

Tensile strength test aims to determine tensile strength of the scaffold. Determination of mechanic characteristic was conducted by cutting the scaffold in dumbbell shape. The data obtained are elastic boundary information (σE), tensile strength, and strain. Based on the measurement, voltage curve and strain were obtained. Strain strength is a maximum pull which is achievable until the material can survive and tear. Material's stiffness can be analyzed through the calculation of the young modulus which is the ratio between tensile strength and strain. The mechanical test was performed using Autograph tool. From the tensile strength, a value of 1,425 MPa for tensile strength was performed from sample A /control. The highest value of tensile strength was refer to control sample. The tensile strength value of samples are sample A 1,425 $\pm$ 0,0025, sample B 0,082 $\pm$ 0,0203, sample C 0,732 $\pm$ 0,0015, sample D 0,475 $\pm$ 0,0057, sample E 0,620 $\pm$ 0,0058. While the values of tensile strength in the sample B,C,D, E with *Aloe vera* and snail mucus concentration 0,07%AV: 0,07% SM; 0,15%AV:0,15% SM; 0,07%AV :0,15% SM; 0,15% AV: 0,07% SM subsequently, as illustrated in Figure 4.

Sample B has lowest tensile strength value due to the big pore size. Previous research by Angulo dan Sobral, *scaffold* with addition *snail mucus* has lower *tensile strength* compare with control. This might be related with functional group C-O and –COOH. Type

of polymer and ionic characteristic could yield lower molecules interaction and alter the elasticity [14].
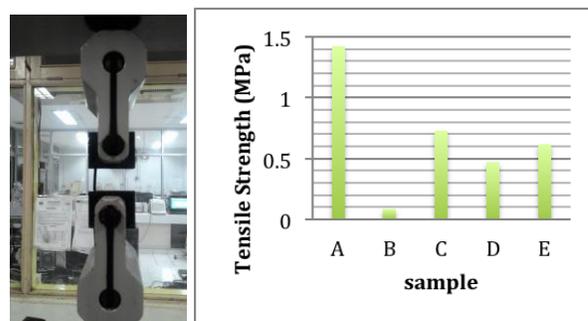

Figure 4 (a) Tensile strength test process; (b) Result tensile strength test Samples A (0%AV, 0% SM), B (0,07%AV, 0,07% SM), C(0,15%AV, 0,15% SM), D(0,07%AV, 0,15% SM), E (0,15% AV, 0,07% SM)

Swelling Test Analysis

Swelling test aims to determine the ability of water retention from scaffold, by soaking the samples in Phospate Buffered Saline (PBS). Analyses of swelling were performed in triplicate/three times. At the first, second, seventh, and fourteenth day, the samples are weighted and calculated to obtain swelling percentage, as illustrated in Figure 5. The swelling value of samples are sample A-E in 1st day 604% $\pm$ 1; 278 $\pm$ 1,15; 263$\pm$ 1,15; 259 $\pm$ 0,58; 261 $\pm$ 0,58 subsequently, in 2nd day 579 $\pm$ 1; 341 $\pm$ 0,58; 318 $\pm$ 1,53;313 $\pm$ 0,58; 314 $\pm$ 0,58 subsequently, in 7th day 563 $\pm$ 0,58; 381 $\pm$ 1; 338 $\pm$ 1,73; 332 $\pm$ 1,15; 334 $\pm$ 1,53 subsequently, in 14th day 560 $\pm$ 1,55; 520 $\pm$ 1; 339 $\pm$ 1; 336 $\pm$ 1,15; 337 $\pm$ 1,15 subsequently. In this research, the swelling value reached 200-520% which was close to the swelling value in the research of Rose *et al*. at 2013 [23], which had reached 300-400%. The samples contained *Aloe vera* recorded lower swelling value compare with sample without *Aloe vera*. It might be caused by the affinity and good interaction between the matrix and scaffold with *Aloe vera* which can actually decrease water binding capability. The water uptake of scaffold decreased with increase in the concentration of the crosslinker used. The equilibrium swelling of scaffold also decreased with increase in the content of gelatin as crosslinker. Short crosslinking time resulted in material, which was characterized by a rapid increase in the swelling of material in the first 4 hours, followed by a collapse of network structure. Increasing the crosslinking time resulted in a decrease in the swelling capacity of the material because of the formation of a higher density of crosslinking points in the network structure [24].
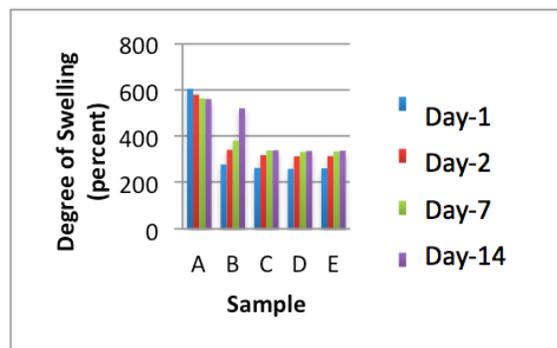
Figure 5 (a) Swelling test results of Samples A (0%AV, 0% SM), B (0,07%AV, 0,07% SM), C(0,15%AV, 0,15% SM), D(0,07%AV, 0,15% SM), E (0,15% AV, 0,07% SM

Degradation Test Analysis

Degradation is the physical (or sometimes chemical) deterioration of a compound due to external chemical exposure or ambient conditions. Degradation test aim is to discover how long it takes for a sample to decompose in the body, so that the cell could produce an extracellular matrix [25]. Therefore, degradation character of the scaffold is essential to select and design biomaterials, as well as for the long-term success of tissue engineering [16].
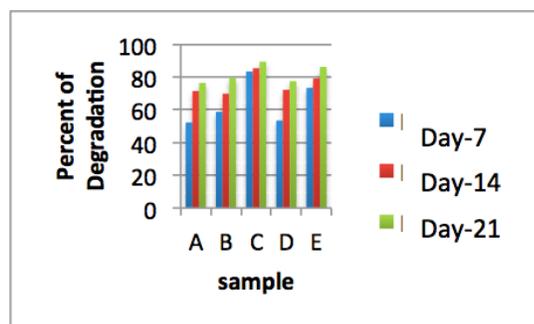

Figure 6 (a) Degradation test result of Samples A (0%AV, 0% SM), B (0,07%AV, 0,07% SM), C(0,15%AV, 0,15% SM), D(0,07%AV, 0,15% SM), E (0,15% AV, 0,07% SM)

The samples are immersed in Phosphate Buffered Saline (PBS) solution resembling body fluids. Then, a percentage of weight less was calculated at a given time, such as the 7th, 14th and 21st day. The value of degradation of sample A – E in 7th day are 52,16 $\pm$ 1,06; 58,67 $\pm$ 0,89; 83,29 $\pm$ 1,01; 53,4 $\pm$ 1,07; 73,3 $\pm$1,29 subsequently, in 14th day 71,37 $\pm$ 1,4; 69,72 $\pm$ 1,8; 85,27 $\pm$ 1,77; 72,14 $\pm$ 1,42; 79,04 $\pm$ 1,11 subsequently, 1n 21st day 76,31 $\pm$ 1,06; 79,3 $\pm$ 1,66; 89,26 $\pm$1,13; 77,41 $\pm$ 1,07; 86,10 $\pm$ 1,51 subsequently. Analyses of degradation were performed in triplicate/three times. Based on Figure 6, after the 21st day of incubation process, scaffold was degraded over 70% to 90%, proving its biodegradable character in a body fluid-like circumstance. Based on the degradation test results,

on the 21st day, the samples are not 100% degraded, they met the standards of scaffolds for burns cases, because skin regeneration period in burns cases needed 21 days or more to recover [26] so good scaffold should not 100% degraded before the 21st day.

4.0 CONCLUSION

Characteristics of gelatin / chitosan with *Aloe vera* and *Achatina fulica sp* mucus biocomposite in skin tissue engineering in the burn case are showed in FTIR that there has been a bond between the chitosan with gelatin and aloe vera with the *Achatina fulica sp* mucus. While the pore size, porosity and swelling value of biocomposite scaffold meet up with application standard. Eventhough the tensile strength s are still need to optimized. The degradation time of this biocomposite scaffold is 21 days, compatible with standard. Gelatin-chitosan Biocomposites, along with the addition of *Aloe vera* and snail mucus extracts is potentially used as scaffold for tissue engineering in burns cases. Sample with 0,15% Aloe vera and 0,07% *Achatina fulica sp* mucus

Acknowledgement

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