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Antibacterial Activity of Sugarcane Bagasse Nanocellulose Biocomposite with Chitosan Against *Eschericia coli*

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Abstract

Antibacterials have been used to treat infectious diseases in both humans and animals since 1929. Along with their use, there is resistance to some antibacterials. 43% *Escherichia coli* is resistant to various types of antibiotics. Therefore, research related to development of antibacterial material is needed. Nanocellulose has received a lot of attention on its application of antibacterial material support. Meanwhile, Chitosan is an antibacterial biopolymer with a brittle structure. Therefore, nanocellulose is added to chitosan film to increase its structural stability. In this study, nanocellulose was extracted from sugarcane bagasse through a combination method of sulfuric acid hydrolysis with ultrasonic waves. The effect of adding nanocellulose to chitosan mechanical properties was investigated. Scannin Electron Microscopy test showed that there were differences in morphology between nanocellulose, chitosan, and nanochellulose-chitosan biocompites. The result of X-Ray Diffraction and Fourier-transformed infrared spectroscopy analysis showed that biocomposites was successfully formed. The average size of nanocellulose particle was 132,67 nm. Nanocellulose-chitosan biocomposites with a ratio of 10:2 have the best antibacterial activity against *Eschericia coli* than other biocomposite ratios.

Keyword: Antibacterial activity, Nanocellulose-chitosan, *Eschericia coli*, Sugarcane bagasse, Ultrasonication

1. INTRODUCTION

Antibacterials have been used to treat infectious diseases in humans and animals since 1929. Along with their use, there is resistance to several antibacterials that can threaten the health of millions of lives. The rate of bacterial resistance to drugs is increasing rapidly. The Antimicrobial Resistance In Indonesia (AMRIN-Study) at 2011 proved that of 2,494 individuals spread throughout Indonesia, 43% of *Eschericia coli* were resistant to various types of antibiotics, including ampicillin, cotrimoxazole, and chloramphenicol (Hadi et al., 2013). Therefore, research related to the development of antibacterial ingredients is needed. Nanocellulose has received a lot of attention in its application as an antibacterial material support (Juanjuan Li et al., 2018). The antibacterial properties of nanocellulose are due to their physical properties, excellent biological properties, and special surface chemical properties. Nanocellulose has a large number of hydroxyl group so that is has stable structure in water (Phanthong et al., 2018).

Several studies have proven the ability of nanocellulose as an antibacterial support material (George & Sabapathi, 2015; Xu et al., 2019). Nanocellulose can be biocomposite with natural materials to increase its uselfuness (Gunathilake et al., 2017; Hänninen et al., 2018; Oksman et al., 2016; Szymańska-Chargot et al., 2019). Nanocellulose can increase the antibacterial activity of chitosan against *B. cereus, Salmonella typhimurium, S. aureus, Eschericia coli* (Abdul Khalil et al., 2016). Chitosan is a biopolymer compound produced from chitin of crustacean class, such as shrimp and crab, wich can be used as food packaging

material because it is easily degraded, edible film, antioxidant, and antibacterial (Cheung et al., 2015). Chitosan polymers are easily brittle so that nanocellulose is combined to increase the stability of its structure (Szymańska-Chargot et al., 2019).

Nanocellulose can be extracted from agricultural waste, one of which is sugarcane bagasse (Rahmi et al., 2017; Saputri et al., 2018). Nanocellulose extraction methods from bagasse that have been successfully carried out include the high pressure mechanical method (Jihua Li et al., 2012), the enzymatic hydrolysis method (Ribeiro et al., 2019), the acid hydrolysis method (Wulandari et al., 2016), and the ultrasonication method (Shahi et al., 2020). The nanocellulose extraction method used in this study is a combination of the acid hydrolysis method with ultrasonic waves. Optimizations of Nanocellulose-Chitosan ratio in biocomposite were carried out in this study to obtain the correct material parameters with high antibacterial activity against *Escerichia coli*.

The resulting biocomposite then characterized its physical properties using several instruments. X-ray diffraction (XRD) analysis was used to characterize the crystalline structure of nanocellulose and biocomposite. Fourier-transformed infrared spectroscopy (FT-IR) analysis was used to characterize the chemical composition of extracted cellulose and nanocellulose that verifying the removal of lignin and hemicellulose during cellulose extraction process from sugarcane bagasse. Scanning Electron Microscopy (SEM) was used to characterize morphology of sugarcane bagasse, nanocellulose, and biocomposite. Particle size analysis (PSA) was used to determine particle size of nanocellulose.

2. MATERIAL AND METHOD

Instrumentation and Materials

The material used in this research are Sugarcane bagasse gathered in Sidoarjo (East Java, Indonesia) were used in this study. The chemical reagent use were NaOH (p.a Merck), NaClO (p.a Merck), H_2SO_4 (Merck), Filter paper (Whatmann No.44), demineralized aqua (Bratachem), *Eschericia coli* isolate from Institue of Tropical Disease-Universitas Airlangga (ATCC 25922), Natrium Agar Media (Merck), Dimethyl sulfoxide (DMSO Merck). The instruments used in this researxh are glasses equipment, ultrasonicator, Oven (DGG 9053A), anaytical balance (Ohaus px224/E), sentrifuge, microplate reader, Laminar Air flow, Autoclave. Morfology of biocomposite was analyzed by Scanning Electron Microscopy (SEM HITACHI FLEXSEM 1000). Functional group of biocomposite was analyzed by flexible benchtop FTIR spectrometer Cary 360. The biocomposite crystalline phase was analyzed by X-ray diffraction (XRD Philip analytical). Nanocelullose particle size was analyzed using particle size analysis (PSA Horiba Scientific SZ-100).

Isolation nanocellulose form Sugarcane Bagasse

The nanocellulose isolation method used was (Mandal & Chakrabarty, 2011) modified method using an ultrasonicator. Clean and dry sugarcane bagasse (SB) is cut into small pieces and chorused into finer powder, then oven at 60°C for 16 hours. The dry bagasse is put into the Erlenmeyer flask, then 250 mL of 17,5% w/v NaOH is added. The mixture was ultrasonicated for 2 hours at 70 ° C. Furthermore, the mixture is then filtered. The residue that has been produced is then bleached with a 0.7% v / v NaClO solution, then the mixture is sonicated for 2x1 hours. The residue produced during the bleaching process is washed with distilled water. The residue obtained was then dried in an oven at 60 ° C to obtain cellulose (C-SB). A total of 5 grams of C-SB was reacted with 25 mL of 45% H_2SO_4. Hydrolysis was carried out with the aid of an ultrasonicator at a temperature of 70°C for 90 minutes. After that the mixture was neutralized with 28 mL of 0.5 M NaOH and washed with distilled water until the pH was neutral. Then the mixture is centrifuged to obtain nanocellulose (NC-SB). The NC-SB was then dried at 60°C until constant weight (Xu et al., 2019).

Biocomposite preparation

Preparation of chitosan-nanocellulose (CHIT/NC-SB) biocomposites using the method belonging to Szymańska-Chargot et al., (2019) with modifications on the membrane formation process. Chitosan was dissolved in 2% acetic acid to obtain 3% w/v chitosan solution. Furthermore, the nanocellulose was cultured in 3% chitosan solution with various mass ratios of CHIT:NC-SB for 8 hours at 45 ° C (Table 1).

Table 1. Mass ratio of Nanocellulose-Sugarcane Bagasse and Chitosan in biocomposite

Biocomposite	Mass ratio CHIT:NC-SB
0	10:0
1	10:1
2	10:2
3	10:3
4	10:4
5	10:5

Then the mixture is poured into 7,5 cm x 10 cm acrylic molds to form a membrane. Furthermore, the biocomposite membrane was dried for 72 hours. The biocomposite obtained was then characterized. The characterizations carried out by SEM, FT-IR, XRD.

Antibacterial activity test against *Eschericia coli*

Antibacterial activity test of nanocellulose-chitosan biocomposites was carried out on *Escherichia coli* as gram-negative bacteria. Antibacterial activity test was carried out in triplo, Chloramphenicol as a positive control and 10% DMSO as a negative control. Antibacterial activity test was carried out using the well diffusion method. After the Natrium Agar media in the petri dish solidified, 50 bacterial suspension was inoculated and evenly spread on the surface of the media using cotton buds. Furthermore, a well was made in a petri dish with a diameter of 7 mm for each well. The biocomposite with various ratio of concentration was cut to a size of 7x7 mm. Futhermore, Each of the test biocomposite were placed into the well, then incubated for 24 hours at 37°C and observed the formed clear zone. The diameter of the zone of inhibition was measured using a caliper (Amanda et al., 2020).

3. RESULT AND DISCUSSION

Ectraction of Nanocellulose from Sugarcane Bagasse

Extraction of nanocellulose from bagasse consists of 3 stages, alkali treatment, bleaching, and acid hydrolysis. All these steps take place with the help of an ultrasonicator. Starting from raw material (SB), the fisrt stage of extraction was alkali treatment or delignification using NaOH to remove lignin from bagasse. In this process, fiber development occurs so that the hemicellulose, mineral salts, and ash are lost, and produce brownish yellow pulp (Sheltami et al., 2012). The second stage of extraction was a bleaching treatment using 0,7% NaOCl to obtain white cellulose. The third stage of extraction was acid hydrolysis using 45% H_2SO_4. Strong acids can remove the amorphous part of cellulose chain (Effendi et al., 2015). The color of powder changed from brown yellow (Figure 1.a) to light brown (Figure 1.b) after alkali treatment and became white after bleaching treatment (Figure 1.c). After hydrolysis stage, the colour of material changed from white to brown (Figure 1.d). NC-SB produced from 60 grams of bagasse is 6.890 grams, so the yield of nanocellulose isolation from bagasse is 11.483%. The average size of nanocellulose particle was 132,67 nm.

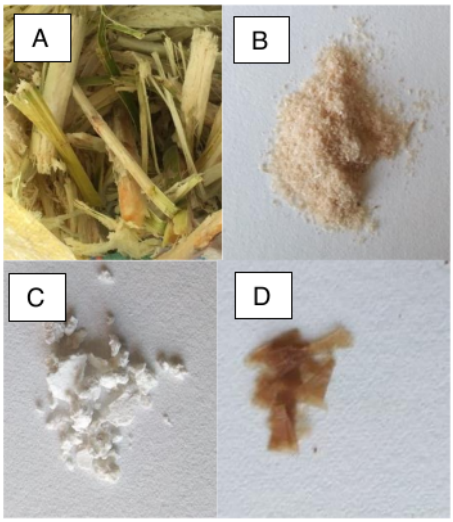


Figure 1. a)Sugarcane bagasse (SB); b) SB After alkali treatment; c) Cellulose; d) Nanocellulose.

Preparation of Nanocellulose-Chitosan Biocomposite

The results of NC-SB/CHIT biocomposite production at various concentration ratios indicated that the more chitosan ratio in biocomposite, the higher the viscosity of the biocomposite mixture. This causes biocomposite micture to rapidly aggregate during the membrane imposing process so that the surface of the biocomposite membrane is not

homogeneous. The temperature during the stirring of mixture and drying of the membrane plays an important role because it can cause sublimation and phase separation so that the membrane formed will agglomerate and make the membrane porous (Hu et al. 2016).

After the biocomposite mixture was homogenous, then the mixture was printed on acrylic 7.5 cm x 10 cm using the casting method. The membrane will form after being heated at 60°C for 72 hours. It can be seen that the higher the chitosan concentration ratio, the lower the transparency of the biocomposite membrane and the color of the bicomposite membrane will become increasingly yellow (Figure 2).

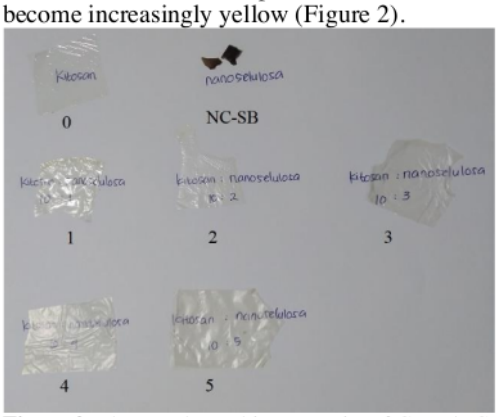


Figure 2. The membrane biocomposite of CHIT/NC-SB

Antibacterial Activity of Nanocellulose-Chitosan against *Eschericia coli*

Inhibition test of *Eschericia coli* bacteria by nanocellulose-chitosan biocomposite can be seen from the formation of clear zones (Figure 2). The clear zone is an area where E*scherichia coli* is not covered, so this area is expressed as the area of bacterial inhibition. The area of inhibition indicates the effectiveness of biocomposites in inhibiting bacteria. The wider the clear zone, the better the ability of biocomposite to inhibit bacteria (Szymańska-Chargot et al., 2019).

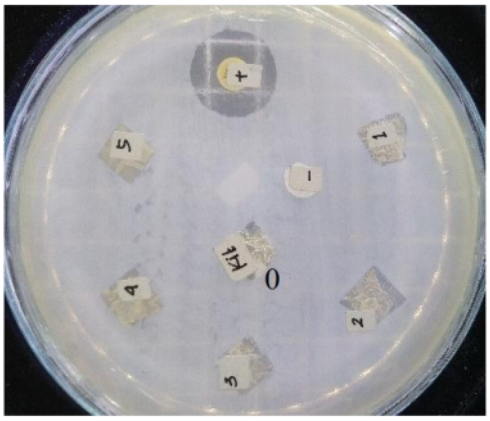


Figure 2. Nanocellulose-Chitosan Biocomposite Inhibition Test Against *Eschericia coli*.

The results showed the most optimum area of inhibition is on the biocomposite membrane with a ratio of 10:2, namely the area of inhibition of 8.5 mm^2 (Table 2). While the area of chitosan inhibition power was 7 mm^2. From these results, the addition of nanocellulose was proven to be able to increase the ability of chitosan to inhibit *Eschericia coli* by 21.43%. The biocomposite mixture is thought to be able to inhibit microbes by destroying the peptidoglycan structure in the cell wall and denaturing proteins, resulting in enzyme deactivation. The deactivated enzyme include phosphodiesterase, isocitrate dehydrogenase, Glucose-5-phospate Dehydrogenase, NADH oxidase, glutamate-pyruvate aminotransferase AlaB, alanin transaminase (Calcott, 1982; Kim et al., 2010; Nieß et al., 2019). This is because the process of inhibiting the growth of microorganisms is generally caused by several things including the presence of disruptive compounds on the cell walls, causing increased cell membrane permeability resulting in loss of cell components, inactivity of enzymes in cells, and the process of destruction or damage to genetic material.(Kirani Paramita et al., 2014).

Table 2. The inhibition zone test against *Eschericia coli*

Biocomposite	**The area of inhibition (mm^2)**
0	7.00 ± 0.05
1	7.20 ± 0.05
2	8.50 ± 0.05

3	7.00 ± 0.05
4	7.00 ± 0.05
5	7.00 ± 0.05
-(DMSO)	0.00 ± 0.05
+ (Chloramphenicol)	78.5 ± 0.05

On the other hand, the addition of chitosan concentration did not show an increase in the area of inhibition power. This could be due to the limited ability of chitosan to bind nanocellulose. Based on the chemical structure of nanocellulose and chitosan, the bonds that occur in biocomposite is strong hydrogen bonds and between ammonium and hidroxyl group of chitosan and Hidroxyl group of Nanocellulose. Not all nanocellulose can be bound by chitosan because of the difficulty of dissolution nanocellulose and chitosan (Abdul Khalil et al., 2016). The Biocomposite 2 with mass ratio 10:2 was chosen to be the most optimum composition and then tested its mechanical and morphological properties.

Membrane Biocomposite Thickness

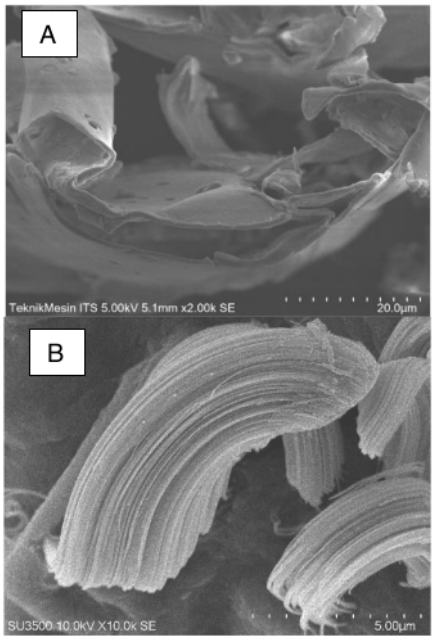
The biocomposite membrane thickness test was measured using a micrometer with a minimum scale of 0.01 mm. Due to the very small thickness of the membrane, to facilitate measurement, eight membranes were stacked and measurements were made at five different points. The thickness measurement results are then divided by eight and averaged. Based on the measurement results, it is known that the membrane thickness with the ratio NC-SB/CHIT (10: 2) is 0.0355 mm ± 0.005 mm, while the membrane thickness with the ratio NC-SB/CHIT (10: 3) is 0.0386 mm ± 0.005 mm. This shows that the higher the chitosan concentration in the bicomposite, the thicker the membrane formed. This is influenced by the presence of biocomposite aggregation factors during the membrane printing process. In addition, this increase in thickness is due to the addition of mass of chitosan biopolymer which binds to nanocellulose which causes the thickness of the plastic to increase (Table 3).

Table 3. The Biocomposite Membrane Thickness

Biocomposite	Tichkness (mm)
0	0.0135 ± 0.005
1	0.0235 ± 0.005
2	0.0255 ± 0.005
3	0.0287 ± 0.005
4	0.0302 ± 0.005
5	0.0312 ± 0.005

Morfology of Biocomposite

Figure 3A shows the morphology of bagasse at 2,000x magnification, Figure 3B shows the morphology of nanocellulose at 10,000x magnification, Figure 3C shows the morphology of chitosan film and Figure 3D shows the surface morphology of the biocomposite nanocellulose-chitosan membrane at 10,000x magnification. From Figure 3A to Figure 3B, It shows the surface of sugarcane bagasse was smoother than nanocellulose because the outer layer of sugarcane bagasse have noncellulosic layer, shuch us pectin, lignin, wax, and hemicellulose that acted as cementing materials to hold the cellulose fibers in bundles (Chowdhury & Hamid, 2016). The dimension of fiber was decrease because of the amorphous region removal in Cellulose. Ultrasonication in the presence of sulfuric acid can hydrolized the nanocellulose amorphous region.



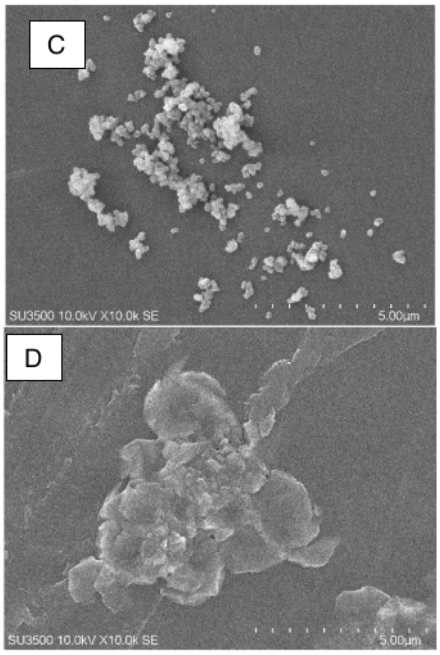


Figure 3. Micrograph of (A) Sugarcane Bagasse; (B) Nanocellulose; (C) Chitosan film (D) Nanocellulose-Chitosan biocomposite (10:2)

Morphological differences of chitosan and chitosan-nanocellulose biocomposite film can be seen on Figure 3C and 3D at 10,000x magnification. In the chitosan layer, separate granules are visible, while in the chitosan-nanocellulose biocomposite, the grain structure is more dense. This shows that the addition of nanocellulose to the chitosan layer can affect the stability of the layer structure so that it can affect the mechanical properties of the layer. Therefore, the increase in antibacterial activity in these biocomposites can be caused by the structural stability of the film layer.

FT-IR Analysis

FT-IR analyisis was used to characterize the effect of nanocellulose addition on chitosan films. The shifts of FT-IR bands are usually related to interaction between molecules. The spectra of pure nanocellulose and chitosan were used as references. The spectra of Chitosan-nanocellulose blend and Nanocellulose were shown to highlight differences among the Sample structures (Figure 4). The nanocellulose spectra contain typical bands for cellulose. The region between 3,600 and 3,200 cm^{-1} is assigned to O-H stretching vibration with the maximum %T at 3300 cm^{-1} related to O-H vibration due to hydrogen intramolecular bonding (Szymańska-Chargot et al., 2019). The region 3,000-2,800 cm^{-1} is related to -CH and -CH_2 stretching vibration. The region 1,500-1,250 cm^{-1} is related to -CH deformation and -OH out-of-plane bending vibration (Li and Renneckar 2011; Zhbankov et al. 2002). The region between 1,180 and 800 cm^{-1} has the same pattern for nanocellulose and chitosan film due to their similarity of molecular structure. This region is also sensitive for the C-O and C-C stretching vibration (Szymańska-Chargot et al., 2019). The main characteristic feature is the presence of bands at 1610 cm^{-1} (overlapped by water band at 1640 cm^{-1}) related to C=O stretching vibration, while bands at 1740 cm^{-1} are related to ester stretching vibration. This results in cellulose oxidation as an outcome of bleaching process with NaOCl (Li and Renneckar 2011; Szyman´ska-Chargot et al. 2019).

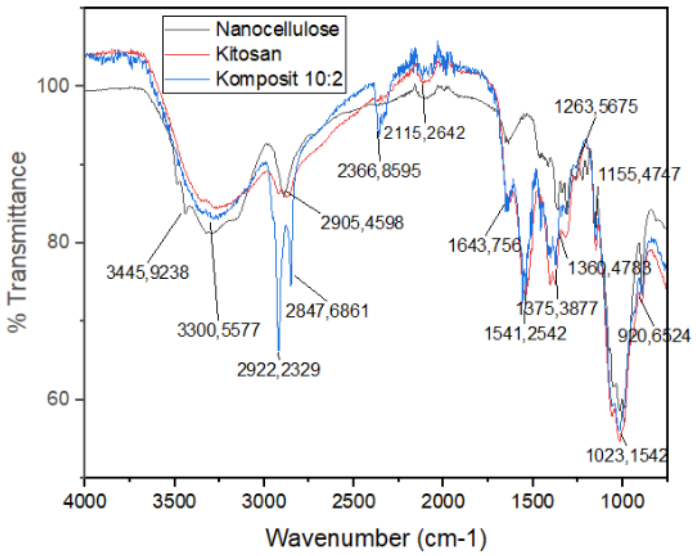


Figure 4. FT-IR Spectra of Chitosan, Nanocellulose, and Biocomposite of Chitosan/Nanocellulose

There are some differences on FT-IR spectrum of biocomposite and chitosan. There was an increase in% T in the area between 2,900-2,800 cm^{-1} at biocomposite spectrum. This indicates that there is an increase in the number of -CH and $-CH_2$ vibrations originating from -CH and $-CH_2$ nanocelluloses. In addition, the biocomposite spectrum has a band in the 2,366 region which indicates a conjugated C = C bond, while the other two spectra have no band in that region. A compound with the conjugated C = C double bond has high reactivity with a stable structure. These properties are due to the fact that the electrons in the phi bond can resonate with the conjugation of the double bond at C = C (Warren, 2008). Therefore, the presence of this conjugated C = C double bond can increase the antibacterial activity of a material.

XRD Analysis

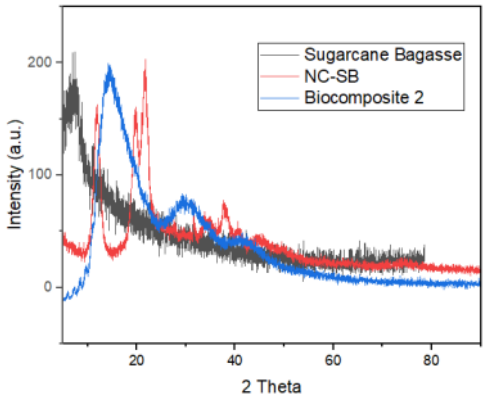


Figure 5. Diffractogram of NC-SB and Biocomposite 2

Figure 5 shows the XRD patterns for sugarcane bagasse and nanocellulose (NC-SB). All of XRD patterns showed peaks around 2θ of 5-22° indicating the typical cellulose I structure. The only difference is slight intensity change in the peaks that indicating some change in the crystallinity inex of samples (Subramanian et al., 2005). The NC-SB peak at 5-25° more defined than Sugarcane Bagasse peak. This is indicated that acid hydrolisis and ultrasonic wave can increase cristanillity index of cellulose. During

the acid hydrolysis process, hemicellulose and lignin were dissolved and the remaining pure of cellulose were isolated. The particle can increases peak intensity and give narrower crystalline peaks (Rosli et al., 2013). Biocomposite 2 (CHIT/NC-SB) showed different XRD pattern at 2θ range from 5° to 40° that indicates the presence of chitosan in composite. The presence of chitosan shows that biocomposite nanocellulose-chitosan successfully formed.

3.5 % Moisture content analysis

Moisture analysis aims to see the effect of nanocellulose addition on the physical properties of chitosan film (Table 4). Based on the data in table 4, chitosan has the highest% moisture than other materials at 7.75%. With the addition of nanocellulose in the chitosan film, the chitosan moisture decreased to 5.24% (Biocomposite 2). This decrease in% moisture content affects the antibacterial activity of a substance. Bacteria easily grow in places with high humidity. Therefore, in this study the% moisture of the material is thought to affect its antibacterial activity. It is evident that biocomposite 2 has a larger zone of inhibition against the growth of Rschericia coli than chitosan film (Table 2).

Table 4. Moisture content analysis

Sample	% Moisture Content
Sugarcane bagasse	1.47
NC-SB	3.85
Chitosan	7.75
Biocomposite 2	5.24

4. CONCLUSION

Based on the research results, the nanocellulose-chitosan biocomposite membrane was able to inhibit the growth of the *Eschericia coli* bacteria. In addition, the inhibition of the biocomposite membrane (10: 2) was better than the inhibition of chitosan against *Eschericia coli*. SEM characterization shows that the addition of nanocellulose can affect the stability of the layer structure, meanwhile FT-IR sectrum shows that biocomposite has conjugated double bond C=C, A compound with the conjugated C = C double bond has high reactivity with a stable structure, XRD analysis indicating that there is a change in crystallinity index of nanocellulose and biocomposite. Of all three characterization of material, it can be concluded that the addition of nanocellulose on chitosan film can increase the material structure stability so ii can increase their antibacterial activity against *Eschericia coli*.

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