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ENZYMATIC SYNTHESIS AND CHARACTERIZATION OF CELLULOSE STEARATE ESTER FROM BACTERIAL CELLULOSE AND STEARIC ACID USING IMMOBILIZED LIPASE

PEMBUATAN DAN KARAKTERISASI ESTER SELULOSA STEARAT DARI SELULOSA BAKTERIAL DAN ASAM STEARAT SECARA ENZIMATIS MENGGUNAKAN LIPASE AMOBIL

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ABSTRACT

The aims of this study were to determine the optimum conditions of enzymatic esterification between stearic acid and bacterial cellulose using immobilized lipase and to characterize the obtained ester. The optimum conditions were determined by the effects of time and mass ratio of cellulose : stearic acid towards degree of esterification. The esterification was carried out in a heterogeneous system using n-butanol as solvent at 50 °C. The effect of reaction time on esterification degree was conducted by varying incubation times for 6, 12, 18, 24 and 30 hours. The influence of the mass ratio of cellulose: stearic acid to the esterification degree was examined by varying 1: 1, 1: 2, 1: 3, 1: 4, 1: 5 and 1: 6. Products characterizations were determined by analyzing functional groups by Fourier Transform Infra Red (FTIR), determining the degree of substitution (DS) by saponification, swelling index by gravimetric method, and crystallinity by X-Ray Diffraction (XRD). The results showed that optimum condition of esterification was 18 hours of reaction with mass ratio of 1:5. The esterification was confirmed by FTIR spectrum that showed ester carbonyl peak at 1718.46 cm^{-1}. The cellulose stearate had the DS of 0.35 which was more hydrophobic and crystalline than bacterial cellulose.

Keywords: cellulose stearate ester, characterization, enzymatic, immobilized lipase

ABSTRAK

Penelitian ini bertujuan untuk mengetahui kondisi optimum reaksi esterifikasi selulosa bakterial dengan asam stearat secara enzimatis menggunakan lipase amobil dan karakter ester selulosa stearat yang dihasilkan. Kondisi optimum diamati berdasarkan pengaruh waktu dan rasio massa selulosa : asam stearat terhadap derajat esterifikasi. Reaksi esterifikasi dilakukan dalam sistem heterogen menggunakan pelarut n-butanol pada suhu 50° C. Pengaruh waktu reaksi terhadap derajat esterifikasi dilakukan dengan variasi waktu inkubasi selama 6, 12, 18, 24 dan 30 jam. Sedangkan pengaruh rasio massa selulosa : asam stearat terhadap derajat esterifikasi dilakukan dengan variasi 1:1, 1:2, 1:3, 1:4, 1:5 dan 1:6. Karakterisasi produk meliputi analisis gugus fungsi secara spektrofotometri *Fourier Transform Infra-Red* (FTIR), penentuan derajat substitusi (DS) secara saponifikasi, indeks swelling dengan gravimetri, dan kristalinitas dengan *X-Ray Diffraction* (XRD). Hasil penelitian menunjukkan kondisi optimum reaksi esterifikasi terjadi pada waktu inkubasi 18 jam dengan rasio 1:5. Hasil analisis struktur menunjukkan telah terbentuk ester selulosa ditandai dengan adanya puncak khas karbonil pada bilangan gelombang 1718,46 cm^{-1} dalam spektrum FTIR. Ester selulosa stearat yang dihasilkan mempunyai DS 0,35 dan bersifat lebih hidrofob dan lebih kristalin dibandingkan selulosa bakterial.

Kata Kunci: enzimatis, ester selulosa stearat, karakterisasi, lipase amobil

INTRODUCTION

Cellulose is the most abundant biopolymer and classified as renewable natural resources (Panesar, Chavan, Bera, Chand & Kumar, 2009). Cellulose mostly found as constituent of plants. Cellulose derived from plants is normally associated with lignin and hemicellulose so that a separation is needed to obtain pure cellulose. Alternatively, pure cellulose can be obtained from bacterial cellulose. Bacterial cellulose is extracellular polysaccharide that produced by several (some) species of bacteria, such as *Gluconacetobacter* (*Acetobacter)* and the other genus of bacteria (Chawla, Bajaj, Survase, & Singhal, 2009; Czaja, Krystynowicz, Bielecki, & Brown, 2006).

One of the bacterial cellulose that widely studied is *nata de coco*. It is made by using *Acetobacterxylinum* in coconut water as medium with sucrose and acetic acid at pH 4 (Halib, Iqbal, Amin, & Ahmad, 2012). *Nata de coco* and its derivatives have a large scale use to be functional material, for example, as membrane raw material. Utilization of *nata de coco* mainly as its derivatives can be obtained by chemical modification, such as esterification (Puspitasari & Radiman, 2006; Lindu, Puspitasari, & Reinfani, 2010; Qiu & Hu, 2013). This reaction can be performed because of its hydroxyl group.

Esterification is an efficient method for cellulose modification (Nagel & Heinze, 2012). Esterification is carried out by reacting the cellulose and carboxylic acid using a catalyst. The short chain carboxylic acids and long fatty acids can be used. The character of ester cellulose depends on the substituent at the main chain of cellulose. Acetic acid is a short-chain carboxylic acid that mostly used in esterification with cellulose.

Stearic acid is one of long-chain fatty acid which can form an ester with cellulose. Using long-chain fatty acid can resolve a weakness of ester obtained from the short-chain carboxylic acid due to form a product with high DS and increasing the thermal stability. Cellulose stearate ester that synthesized by reacting stearic acid, trifluoroacetic anhydride, and microcrystal cellulose has a degree of substitution 2.99 (Huang, 2012).

During these years, one of the methods to obtain cellulose ester is by reacting cellulose with carboxylic acids such as anhydride and alkanoyl chloride in a homogenous systems using N, N-dimethylacetamide/LiCl or N_2O_4/DMF (Qiu & Hu, 2013). The reaction has limitations because the reagent was toxic and difficult to obtain. Thus, enzymatic esterification can be an alternative method that more favorable.

Enzymes are green catalysts which are biodegradable and those can be operated in mild conditions (Matama & Paulo, 2014)**.** One of the enzymes that used in esterification is lipase from *Mucor miehei* In some applications, the preferred enzyme used in immobilized state to facilitate separation from the product at the end of the process, increase the stability, so it can be reused, being continuous operation, more controllable, and economics (Stergiou et al., 2013).

The activity of immobilized lipase is influenced by several factors including temperature, reaction time, the ratio of reactants, and pH. The optimum condition of lipase in esterification lactose by palmitic acid was achieved at 50˚C and 24 hours of incubation time (Roosdiana, Mardiana, Setianingsih, & Suratmo, 2009). The optimum condition of synthesis some sugar esters (glucose, fructose, sucrose, sorbitol and xylose) with fatty acids (oleic acid, palmitic acid, myristic acid, lauric acid and palm fatty acid) occurred at molar ratio 3:1 (sugar:fatty acid) in 24 hours (Vitisant et al., 2012). Esterification of sorbitol with some fatty acid such as oleic acid, palmitic acid, and lauric acid using *Candida antartica* lipase B was optimum at 40-50˚C in 48 hours (Patil, Usmani, & Meshram, 2014).

This research aimed to determine the optimum condition of esterification bacterial cellulose with stearic acid using immobilized lipase. The observed variable was the influence the reaction time and mass ratio of reactants on the esterification degree. The resulted cellulose stearate was determined its physical and chemical properties.

EXPERIMENTAL SECTION

Material and Instrumentation

Bacterial cellulose, *Nata de Coco Ascaso*, was purchased from Gandaria Street 11 Malang. All chemicals were purchased from MERCK and for analysis grade (chitosan, calcium hypochlorite, stearic acid, NaOH, HCl, KBr, n-butanol, ethanol , sodium tripolyphosphate, acetic acid glacial and phenolftalein). *Mucor miehei* lipase (102 U/g) was purchased from Sigma-Aldrich

Instruments used were a set of glassware, spatula, hot hydraulic press, dry grinder, 100-120 mesh of sieve, burette, stative, aluminum foil, paper filter, analytical balance, oven, FTIR spectrophotometer (Shimadzu) and XRD (PANalyticalXpert PRO).

Procedures

Preparation of Bacterial Cellulose Powder

Nata de coco was cleaned and then soaked in 0.1 M NaOH aqueous solution and washed with running water. *Nata de coco* then was soaked in 0.5% **c**alcium hypochlorite solution and washed again with running water. Each treatment was carried out for 24 hours. *Nata de coco* was pressed at 110 -120 °C for 5 minutes using a hot hydraulic press. The dried *nata de coco* then was ground using a dry grinder. The powder was sieved through 100-120 mesh of a sieve.

Preparation of Immobilized Lipase

Chitosan was weighed 1.25 g and then dissolved in 50 mL of 3 % glacial acetic acid solution. Then, 0.5 g of lipase was added to 5 mL of chitosan solution and homogenized using spatula. The mixture was dropped into 10 mL of 3% sodium tripolyphosphate solution and allowed to stand for 75 minutes then that put in the refrigerator.

Synthesis of Cellulose Stearate

Determination of Optimum Reaction Time

Bacterial cellulose powder and stearic acid each was weighed 0.1 g and put them in vial bottles. Next, 5 mL of n-butanol and 0.1 g of immobilized lipase were added to the mixture. The mixture was incubated at 50 °C with variation of time 6, 12, 18, 24 and 30 hours. After incubation, the mixture was then filtered to obtain the solids and filtrate. The solid (ester) was washed with ethanol and then dried in an oven at 80 ºC. The filtrate was titrated by 0.1 M NaOH aqueous solution. Phenolphthalein indicator was added to observe the color change from colorless to pink. The esterification degree was calculated based on the mass of reacted stearic acid divided by the mass of bacterial cellulose. Each variation of reaction time in this procedure was repeated three times.

Determination of Optimum Mass Ratio of Reactant

Esterification was carried out at optimum reaction time. The mass ratio of bacterial cellulose : stearic acid were 1: 1, 1: 2, 1: 3, 1: 4, 1: 5 and 1: 6 (mass of stearic acid was 0.1; 0.2; 0.3; 0.4, 0.5 and 0.6 g). The procedures were similar with the procedures of optimum reaction time determination.

Characterization of Cellulose Stearate Ester

Analysis of cellulose stearate esters using FTIR was conducted to determine its functional groups. In order to obtain the spectra, a sample pellet was ground with KBr. Transmission was measured at the wave number range 4000 - 400 cm^{-1}.

DS of cellulose stearate ester was determined by saponification in heterogeneous systems (ASTM D871-96, 2004) with some minor modifications. 0,5 g of ester was weighed and added 40 mL of ethyl alcohol (75 %). The flasks were heated, loosely stoppered, for 30 min at 50 to 60 °C. 40 mL of 0.5 N NaOH solution was added to flask and heated again at 50 to 60 °C for 15 min. The flasks were closed tightly and allowed to stand at room temperature for 48 h. The excess NaOH at the end of this time was retitrated with 0.5 N HCl solution using phenolphthalein as the indicator. This procedure was also applied to the blank. DS was calculated by following equation (Bono et al., 2009):

$$A = ((B-C) \cdot D) / E$$
$$DS = (0.162 \cdot A) / (1 - (0.2675 \cdot A))$$

where,
A = milli equivalents of consumed acid per gram of specimen; *B* = volume of consumed HCl in blank; *C* = volume of consumed HCl in sample; *D*= concentration in normality of HCl; *E* = specimen gram used. 162 is the molecular weight of the anhydrous glucose unit and 267.5 is the molecular weight of substituent from stearic acid.

The hydrophilicity of ester was observed by determining the swelling index ester in distilled water as the diffusing agent. The swelling index was calculated by following equation (Caillol et al., 2012):

$$Swelling\ index(\%) = ((Ws-Wd) / Wd) \cdot 100$$

Where, *Ws* and *Wd* are the weights of the swollen bar and the dried bar, respectively.

XRD analysis was performed to determine the crystallinity of cellulose stearate. The crystallinity index iscalculated by dividing the total area of crystalline peaks by the total area under the diffraction curve (crystalline plus amorphous peaks). The crystallinity index of samples, by the following equation (Ciolacu, Ciolacu, & Popa, 2011):

$$Cr.I.(\%) = (Sc / St) \cdot 100$$

Where,
Sc : area of the crystalline domain,
St : area of the total domain.

RESULTS AND DISCUSSION

Optimum Condition of Esterification Bacterial Cellulose with Stearic Acid Using Immobilized Lipase

Among the factors affecting enzymatic esterification reaction are the reaction time and the ratio amount of reactants (Vitisant et al., 2012). Esterification reaction in this study was conducted at the optimum temperature of lipase activity of 50 ºC.

The optimum reaction time for the esterification of bacterial cellulose with stearic acid occurred within 18 hours with esterification degree was 0.17. Figure 1 showed that the increase of esterification degree occurred at 6–18 hours and it decreased at 24 and 30 hours.

The decrease value of esterification degree at 24 and 30 indicated the occurrence of the hydrolysis reaction as a reversible reaction of esterification reaction. This was due to the esterification reaction also produced water as a by product. Increasing the amount of water caused the reaction shift toward the hydrolysis reaction than the esterification reaction. The reaction with the lipase as catalyst in organic solvents are affected by water activity. The yield and the reaction rate decreased due to increasing water activity (Rajendran, Palanisamy, & Thangavelu, 2009). Therefore, it is necessary to control the amount of water to enhance ester.

The esterification reaction to determine the effect of the mass ratio of reactanonthe esterification degree was performed at the optimum reaction time by using variationof the mass ratio of reactantsat 1: 1 to 1: 6. The results of the study are shown in **Figure 2**. The curve showed that the greater the mass of the stearic acid to the mass of bacterial

cellulose, the higher the esterification degree. This is due to the increased mass ratio of stearic acid to the bacterial cellulose, the higher concentration of stearic acid in the reaction system so that the possibility of esterification the greater.

The highest degree of esterification occurred at ratio of 1:6 that was 0.97, but ratio 1: 5 and 1:6 did not show statistically significant differences, therefore it can be concluded that the optimum ratio of reactants was 1: 5. The esterification reaction using lipase occured through Ping-Pong Bi-Bi mechanism (Stergio et al., 2013; Bubalo et al., 2015). Lipase reacts with stearic acid to form acyl-lipase and water, then acyl-lipase reacts with bacterial cellulose to form esters.

Lipase catalyzed esterification begins with the acylation step where the catalytic serine residue in the lipase reacts with a carboxylic acid (stearic acid). The acid forms an ester bond with the hydroxyl group of the serine to produce acyl-enzyme intermediate. In the following deacylation step, a hydroxyl (OH from bacterial cellulose) attacks the acyl-enzyme complex, the product ester is released and the enzyme returns to its original state (Viklund, 2003). At the ratio 1: 5, the formation of an acyl-enzyme has reached to maximum state. The ability of an enzyme to absorb the substrates has been reduced because the enzyme was already saturated by substrates. Consequently, the more stearic acid addition did not affect to the degree of esterification significantly.

The physical and chemical properties of Cellulose Stearate.

FTIR spectrum showed changes in absorption band between bacterial cellulose and ester, either a shift of wave number or the formation of new band on cellulose stearate. Thus, the formed cellulose stearate can be approved towards shift of wave length of functional group.

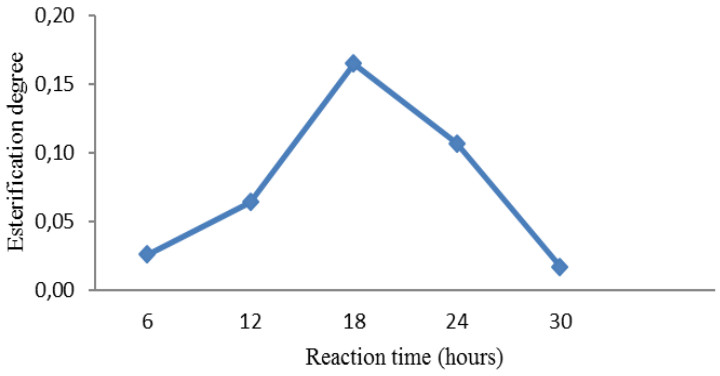

Figure 1. The effect of reaction time to the degree of esterification of cellulose stearate

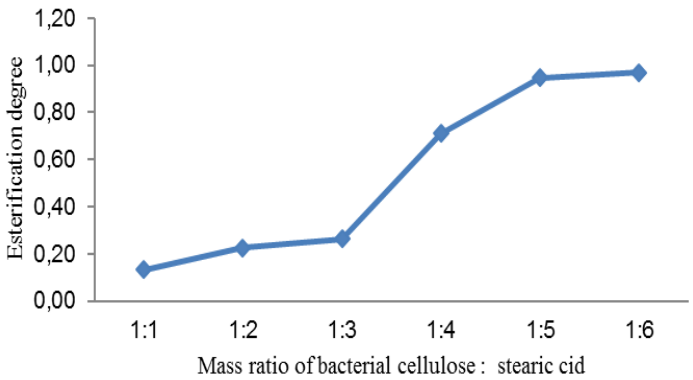

Figure 2. The effect of mass ratio of reactants (bacterial cellulose : stearic acid) to the degree of esterification of cellulose stearate

Cellulose sterate spectrum showed the decrease in the intensity of broad band at 3244.05 cm^{-1} assigned to cellulose –OH vibrations. It indicated that a number of OH groups were substituted. The spectrum of cellulose stearate also showed an increase of the intensities of the characteristic bands of CH bonds alkyl from alipathic acid chain at 2850.59 and 2918.10 cm^{-1} (CH anti-symmetric and symmetric stretching of CH_2 and CH_3). There were two new bands in the spectrum of cellulose stearate (**Figure 3b**). The first band appeared at 1718.46 cm^{-1} (C=O stretching), corresponding to the vibration of carbonyl ester groups. The second band appeared at 717.47cm^{-1} (CH_2 rocking) which was characteristic of at least four linearly connected CH_2 groups (Huang, 2012; Tome et al, 2010). The intensity of C=O for cellulose stearate was not too strong. It was possible due to a small DS and low purity of ester.

The degree of substitution (DS) is the average number of substituted hydroxyl groups for each anhydroglucose unit. Cellulose has three hydroxyl groups that are OH-2, OH-3 and OH-6 . That means the maximum value of the degree of substitution of the cellulose is 3.

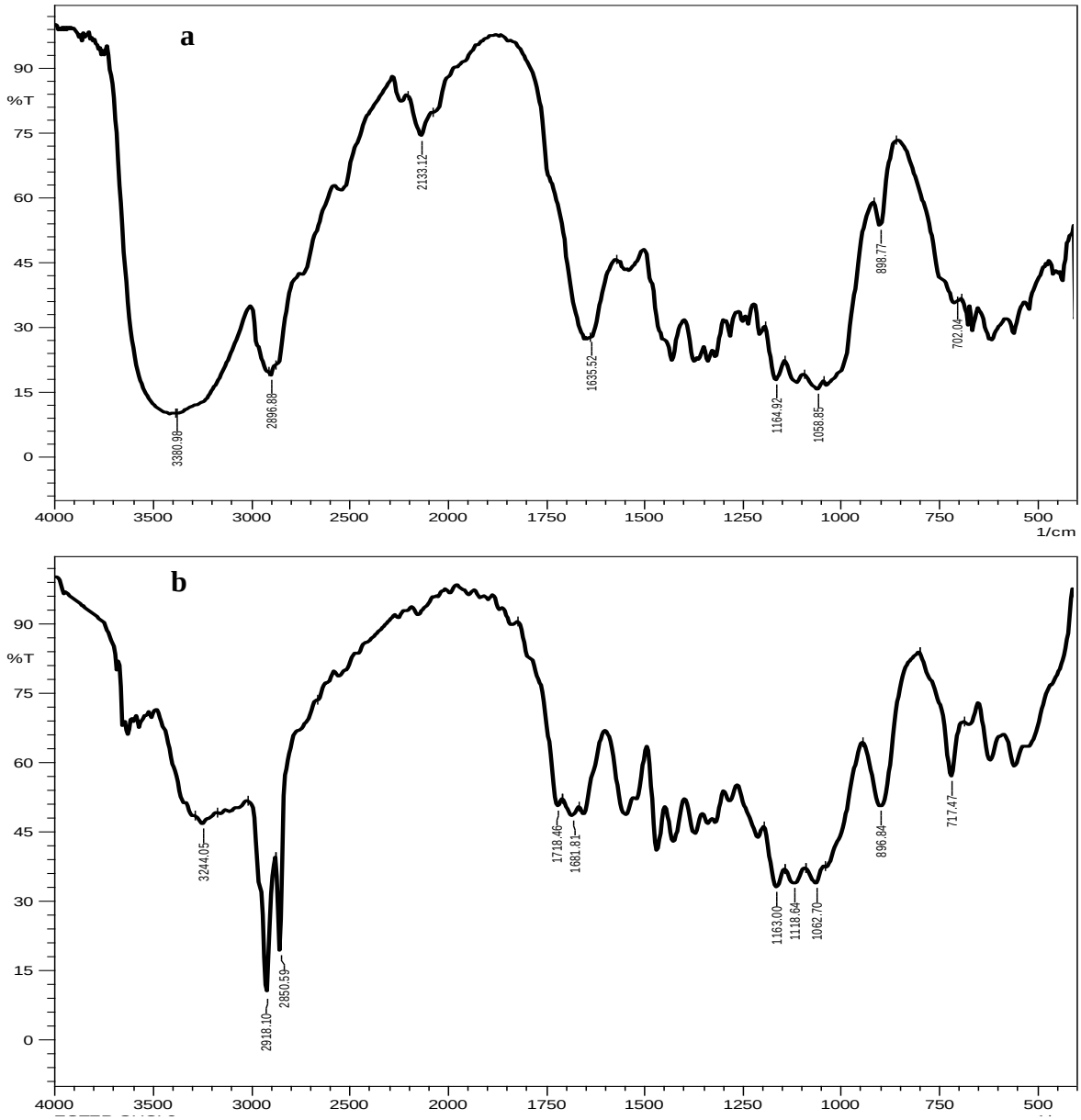

Figure 3. FTIR spectrum **(a)** bacterial cellulose **(b)** cellulose stearate

In this study, DS of cellulose stearate ester was 0.35. It showed that not all of hydroxyl group of bacterial cellulose was substitued by stearic acid. These results corresponded with the results of the analysis of functional groups by FTIR.

Different from the results of enzymatic esterification, cellulose stearate ester made by reacting stearic acid, trifluoroacetic anhydride and microcrystalline cellulose has a degree of substitution 2.99 (Huang, 2012). Possibly, the enzymatic esterification reaction is strongly influenced by the source of lipase. Lipases from different sources have activity and reactivity to the type of alcohol or carboxylic acid. Controlling the amount of water at the esterification system is also important to produce esters with a higher degree of substitution.

Hydrophilicity of cellulose stearate ester was determined by measuring the equilibrium time of swelling and swelling index. Equilibrium time of bacterial cellulose was 5.9 hours with swelling index 315%, while the cellulose ester showed equilibrium time 8.6 hours with swelling index 249%. It showed that the hydrophilicity of cellulose stearate ester was smaller than bacterial cellulose. Cellulose bacterial has a high hydrophilicity because it has many OH groups in its chain while the cellulose ester stearate, OH groups in cellulose ester were replaced with long-chain substituent and reduced its hydrophilicty. This means that the cellulose ester absorbs less water than bacterial cellulose.

The diffractogram of bacterial cellulose and cellulose stearate are shown in **Figure 4**. Diffractogram showed characteristic peaks of cellulose at 2θ 15º and 22.5º (Barud et al., 2007). Crytallinity index of bacterial cellulose and cellulose stearate was calculated based on their amorph and crystalline areas of diffractogram. The crystallinity index of bacterial cellulose and cellulose stearate ester were 57 % and 62 %, respectively.

The more regular the chain of cellulose stearate, the smaller its amorphous regions and the larger crystalline area. It causes a high degree of crystallinity. Cellulose stearate had a higher crystallinity index than bacterial cellulose. It was possible because cellulose stearate had carbonyl group and it formed hydrogen bonding. Hydrogen bonding at the chain of polymer can increase its crystallinity.

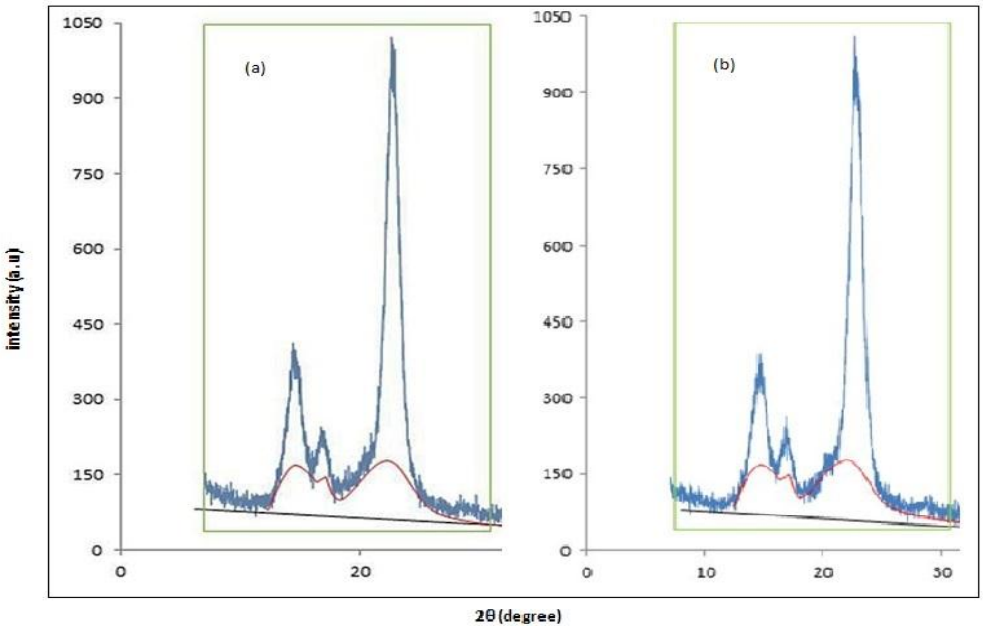

Figure 4. Diffractogram of **(a)** bacterial cellulose **(b)** cellulose stearate

CONCLUSION

Enzymatic esterification of bacterial cellulose with stearic acid was succesfully achieved. The optimum conditions of esterification occurred within 18 hours with a mass ratio of reactants 1: 5. The changes of esterified cellulose properties have been evaluated by ester characterization. Cellulose stearate esters was confirmed by the appearance of shift and new band in FTIR spectrum. Cellulose stearate DS was 0.35. Esterification reduced the hidrophilicity and improved the crystallinity of bacterial cellulose.

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