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Cellulase Pretreatment of *Kappaphycus alvarezii* polymer for Promising Medical Dressing Application

Dyah Rika Ar Rosyidah[1], Dini Ermavitalini[1], Maharani Pertiwi Koentjoro[2], Isdiantoni[3], Ida Ekawati[3], Endry Nugroho Prasetyo[1]

[1]Biomaterial and Enzyme Technology Research Group, Microbiology and Biotechnology Laboratory, Biology Department, Faculty of Natural Science, Institut Teknologi Sepuluh Nopember (ITS), Gedung H Kampus ITS Keputih Sukolilo Surabaya 60111 Indonesia.

[2] Medical Laboratory Technology Study, Faculty of Health, Nahdlatul Ulama University of Surabaya, Jl. Raya Jemursari Surabaya 51-57, Surabaya-Indonesia.

[3]Agrobisnis Department, Faculty of Agronomy, Universitas Wiraraja, Jalan Raya Sumenep-Pamekasan Km. 5 Patean, Sumenep 69451, Indonesia.

Corresponding author: Dr. Endry Nugroho Prasetyo (endry@bio.its.ac.id)

Abstract. Seaweed contains hydrocolloid polysaccharides which are useful for the application of wound dressing. The use of natural ingredients such as seaweed *Kappahycus alvarezii* requires pretreatment before it is applied. Pretreatment of seaweed *Kappaphycus alvarezii* flour was carried out using hydrolyzing enzyme called cellulase. The cellulase (EC 3.2.1.4) is an enzyme which could hydrolyze cellulose polymers thereby breaking β-1,4 glycosidic bond into oligosaccharide and simple sugars. Meanwhile the cell wall of seaweed could as well be degraded partially by cellulase followed by releasing hydrocolloid polymers (carrageenan) from the inner layer of seaweed cell wall. The aims of this study is to determine the effect of enzymatic pretreatment on seaweed so that it can be useful for further research as one of the promising component in wound dressing. The result showed that the reducing sugar decreased in content of 0.5 mg /ml after enzymatic modification at 0.5 hours incubation time. The FTIR spectra showed shifting at several functional groups namely C-H, C-O-C and C=C.

1. Introduction

In the regenerative medicine fields, natural biopolymers are widely used for wounds and burns because of their property among others are biocompatibility, biodegradability and similarity to macromolecules recognized by the human body [1,2]. Several products used as wound dressing made from natural biomaterials among others are hydrogel, hydrocolloid, transparent film etc. Hydrogels are widely used in biomedical applications because various advantages include being able to absorb and release a large amount of water reversibly [3]. In addition, the structure possessed by the hydrogel makes it capable of being used as transport of bioactive molecules such as antibiotics and various drugs to the wound center to accelerate the healing process [4]. The physical properties of hydrogels resemble tissue in living things, such as high water content of up to 70-90%, consistency of soft and supple structures, and a low tendency to absorb proteins present in body fluids [5,6]. Hydrogels also have the potential to become "smart release delivery systems" because they can reduce the potential for denaturation and aggregation of proteins contained therein and can be used as wound dressing [7].

Hydrogel can be made from natural biomaterials. Natural biomaterials are derived from animals, microbials, or plants [2]. Seaweed contains hydrocolloid polysaccharides which are useful for the application of wound dressing. Hydrocolloids in seaweed include agar, alginate, carrageenan and cellulose [8,9,10]. *Kappaphycus alvarezii* is a type of red algae seaweed which contains a lot of kappa carrageenan which can be made into a hydrogel. The gel formed can be used as an injectable matrix to transfer bioactive molecules. Seaweed cell walls are composed of two different layers. The outermost layer is a layer containing cellulose and phospholipid fibers, while the inner layer of the cell wall contains mostly galactant sulfate (carrageenan) polymers [9,11].

The use of polysaccharides in its natural form requires pretreatment first for modification in its structure [12]. Pretreatment can be done physically, chemically or biologically. Physically and chemically pretreatment has several disadvantages including the occurrence of non-specific changes that affect the changes in the structure of the hydrocolloid molecule randomly and uncontrollably [13]. Biological pretreatment can be carried out enzymatically using cellulase to produce a hydrocolloid structure that is better than physical and chemical modification. Cellulase (EC 3.2.1.4) known as the systematic name of β-1,4 glukan-4-glucano hydrolase is an enzyme that can hydrolyze cellulose polymers by breaking the bonds of glycosidic β-1,4 into simple sugars [14]. Another function of cellulase in the pretreatment of seaweed structure is to reduce the constituents of seaweed cell walls which further facilitate the release of carrageenan polymers [15]. The aims of this study is to determine the effect of enzymatic pretreatment on seaweed so that it can be useful for further research as one of the promising component in wound dressing.

2. Materials and Methods

2.1 Materials

Trichoderma harzianum, Potato Dextrose Agar, Potato Dextrose Broth, Urea, Malt Extract, Yeast, ammonium sulfate $(NH_4)_2SO_4$, Potassium dihydrogen phosphate KH_2PO4, Calcium chloride dehydrate $CaCl_2.2H_2O$, Magnesium sulfate Heptahydrate $MgSO_4.7H_2O$, Ferrous sulfate heptahydrate $FeSO_4.7H_2O$, Maganese Sulfate Heptahydrate $MnSO_4.7H2O$, Zinc sulfate heptahydrate $ZnSO_4.7H_2O$, Cobaltous chloride hexahydrate $CoCl_2.6H_2O$. DNS (dinitrosalicylic acid) reagent, citrate buffer, CMC (carboxymethyl cellulose) (Sigma-Aldrich). Bradford reagent, BSA (Bovine serum albumin) (Sigma-Aldrich). *Kappaphycus alvarezii* was obtained from Potteran Island, Sumenep, Madura.

2.2 Cellulase Production and Isolation

Trichoderma harzianum isolates was re-cultured first using Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) media. A number of 10 % inoculum was inoculated in Mendels media containing CMC (carboxymethyl cellulose) to adapt with cellulose as a carbon source and were incubated in rotary shaker at room temperature (±28^oC). Cellulase was produced by *Trichoderma harzianum* through fermentation using Mendels Media containing Urea 0,3 gr/L, malt extract 0,75 gr/L, yeast 0,25 gr/L, $(NH_4)_2SO_4$ 0,3 gr/L, KH_2PO4 2 gr/L, $CaCl_2.2H_2O$ 0,3 gr/L, $MgSO_4.7H_2O$ 0,3 gr/L, $FeSO_4.7H_2O$ 5 mg/L, $MnSO_4.7H2O$ 1,6 mg/L $ZnSO_4.7H_2O$ 1,4 mg/L, $CoCl_2.6H_2O$ 20 mg/L and CMC powder 10 gr/L. Afterwards cellulase was isolated by centrifugation method at 10000 rpm for 10 minutes in cold temperature (4^oC) when the culture in the middle of stationary phase. Supernatant was collected as cellulase to determined the activity and protein of enzyme.

2.3 Enzyme Assays

Enzyme activity was measured using the DNS method (Dinitrosalyclic acid). Digestion solution consisting of 1 ml substrt CMC (carboxymethyl cellulase) 2% in citrate buffer (pH 5; 0.05 M) and 0.01 ml supernatant of crude cellulase extract) which was incubated for 10 minutes at 50^oC. After the end of incubation, the sample was terminated with the addition of 3 ml of DNS reagent, then heated for 15 minutes in boiling water until a color change occurred and immediately cooled. Enzyme activity was measured using a UV-Vis spectrophotometer at a wavelength of 540 nm. Conversion of glucose levels into activity units is determined based on the glucose standard curve using the following formula:

$$Activity\ \left(\frac{\text{Unit}}{\text{ml}}\right) = \frac{\Delta \text{P}/\text{T}}{\text{V}}\ X\ DF$$

Note:

ΔP = Amount of reducing sugar (mg/ml)

T = Incubation time (menit)

V = total volume of enzyme (ml)

DF = Dilution factor

One unit enzyme activity (U) was defined as the amount of enzyme that released 1 µmol of reducing sugar as glucose equivalents min^{-1} [16,17].

2.4 Protein Determination

Protein content of the enzyme was analyzed using Bradford (1976) method with BSA (Bovine Serum Albumin) as a standard protein. First, by making Bradford reagent, 100 mg of Coomasie Briliant Blue G-250 was dissolved in 50 ml of 95% ethanol and added 100 ml of phosphoric acid 85% (w / v). Then distilled water was added to a volume of 1 liter [18] and filtered with Whatman No.1 filter paper.

Protein level of cellulase was determined by reacting 0.1 ml cellulase with Bradford reagent (5 ml). The solution was homogenized using vortex and its absorbance was measured using a UV-Vis spectrophotometer with a wavelength of 595 nm. The protein content of enzymes can be determined based on the standard curve equation BSA (Bovine Serum Albumine).

The standard protein solution is made by weighing Bovine Serum Albumin (BSA) and dissolving it in H2O to obtain a standard BSA solution with various concentrations (w / v). The 0.1 ml BSA solution to be tested was added to 5 ml of Bradford reagent. The solution was homogenized using vortex and measured its absorbance using a spectrophotometer with a wavelength of 595 nm. The absorbance value is made in the form of a standard curve with the equation y = ax + b, where y is the absorbance value and x is the BSA standard protein concentration value.

2.5 Preparation of Seaweed Kappaphycus alvarezii *Flour*

Kappaphycus alvarezii obtained from seaweed cultivation in Potteran Island, Sumenep, Madura. One kilogram of *Kappaphycus alvarezii* seaweed was washed with clean water to remove salt, sand and epiphytes that were attached to the surface of the sample [19]. Washed samples are dried and then mashed and sieved.

2.6 Enzymatic Pre-treatment of Seaweed Flour

The substrate used in the saccharification is seaweed flour. The cellulolytic enzymes used in the saccharification are derived from liquid fermentation by Trichoderma harzianum. Enzymatic pre-treatment of seaweed substrate is done by directly reacting seaweed flour with cellulase. Enzymatic modification of seaweed was performed in 250 mL erlenmeyer flask consisting of 0.5 grams of substrate and 30 mL of citrate buffer (0.05 mol / L pH 5) and added with 0.4 ml of crude cellulase extract. The mixture was then placed in a 150 rpm shaker incubator at 50 ° C with an incubation time of 0.5 hours; 1 hour and 2 hours. And observed reducing sugar using spectrophotometer [21]. At the end of the incubation, the solution is filtered using a filter paper. then the pretreated seaweed flour is dried using an oven with temperature 60^{o}C for 5 hours.

2.7 Reducing Sugar Analysis

The amount of reducing sugar produced by enzymatic hydrolysis was measured using the method of dinitrosalicylyc acid (DNS) [22]. 1 ml sample was mixed with 2 ml of control reagent. The tube is then heated in a boiling water bath for 5 minutes until the color changes and immediately cooled to room temperature then measured absorbance using a spectrophotometer with a wavelength of 540 nm. The amount of reducing sugar was calculated based on the standard glucose curve with mg / ml units [20].

2.8 FTIR analysis

FTIR (Fourier Transform Infrared Spectroscopy) is a method used to identify functional groups of organic compounds [23]. Measurement of functional groups begins with sample preparation. Samples (seaweed powder and modified seaweed meal) were dried by oven and then analyzed using FTIR (Spectrophotometer FTIR BOMEM MB-100) at wavelength 400 - 4000 cm^{-1}. The analysis using FTIR was carried out at the Materials and Metallurgical Laboratory, Faculty of Industrial Engineering, ITS, Surabaya to determine the change of the number of functional groups on cellulose including hydroxyl, carboxyl and ether groups in the sample before and after enzymatically pretreatment.

3. Result and Discussion

3.1 Cellulase Production and Isolation

Cellulase was produced through a fermentation technique by *Trichoderma harzianum* in Mendels medium which contains CMC (carboxymethyl cellulose) as a carbon source. Cellulase was isolated after the fermentation period based on the growth curve to know to know the right time for enzyme isolation. The growth curve of *Trichoderma harzianum* isolates in the Mendels medium (Figure 1) consists of a phase of lag (adaptation), the exponential phase is the optimal growth phase of microorganisms because of the availability of abundant amounts of nutrients. Stationary phase is a condition that is characterized by the amount of growth equivalent to the number of deaths of microorganisms due to nutrients in the medium begins to thin out so that residual product accumulation can inhibit growth [16].

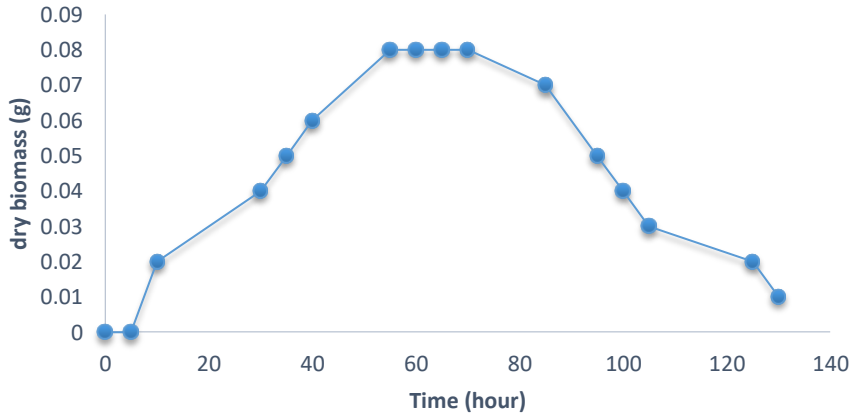

Figure 1. Growth curve of *Trichoderma harzianum* in Fermentation Medium

Lag phase of *Trichoderma harzianum* starts from zero to sixth hours since the starter of inoculum has been acclimatized in the medium containing cellulose as a carbon source. Followed by the log phase at sixth to fifty fourth hours when the isolates growth quickly. The stationary phase of the isolates showed at fifty fourth until sixty sixth hours which indicates that there was no growth of micoorganisme because the rates of isolate's growth and death were balanced. Enzymes were isolated in this phase because the cellulase enzyme optimally accumulates in the culture medium [25]. Cellulase as a secondary metabolites have been produced and induced by the activation of carbon catabolite repression that produce extracellular enzymes which would hydrolyze cellulose in order to fulfil the depleted carbon requirements [16,25].

3.2 Enzyme activity and Protein Content of Cellulase

The cellulase crude extract produced has a cellulolytic activity of 659.95 U / ml with a total protein content of 0.057 mg/ml. Cellulase *Trichoderma harzianum* is most likely a collection of cellulolytic enzymes that are commonly endo-1,4-β-glukanase, exo-1,4-β-glukanase and β-glucosidases [26]. Cellulase known as systematic β-1,4 glukan-4-glucano hydrolase is an enzyme that can hydrolyze cellulose by breaking the glycosidic link β-1,4 in cellulose, cellodectrin, selobiosa, and

other cellulose derivatives into simple sugar or glucose . Cellulose degradation by enzymes produced by fungi involves synergistic action of three classes of enzymes, exoglucanase, endoglucanase, and glucosidase [27].

Endo-1,4-β-glucanase attacks amorphous regions of cellulose randomly and forms considerable non-reducing ends which facilitate exogluconase to work easily. Exo-1,4-β-glucanase hydrolyzes the crystal region of cellulose by releasing two units of glucose. The cooperation of these two enzymes results in smaller saccharide units (cellobiose). β-glucosidases converts cellobiose, the main product from the mixture of endo and exo-glucanase to glucose [27].

3.3 Level of Reducing Sugar from Enzymatic Pretreatment

Yield of reducing sugar from the enzymatic hydrolysis process was analyzed using the 3-5-dinitrosalicylic acid (DNS) method [22]. The results of measurement of reducing sugars during enzymatic hydrolysis using crude enzyme *Trichoderma harzianum* isolates on seaweed substrates are shown in Figure 2.

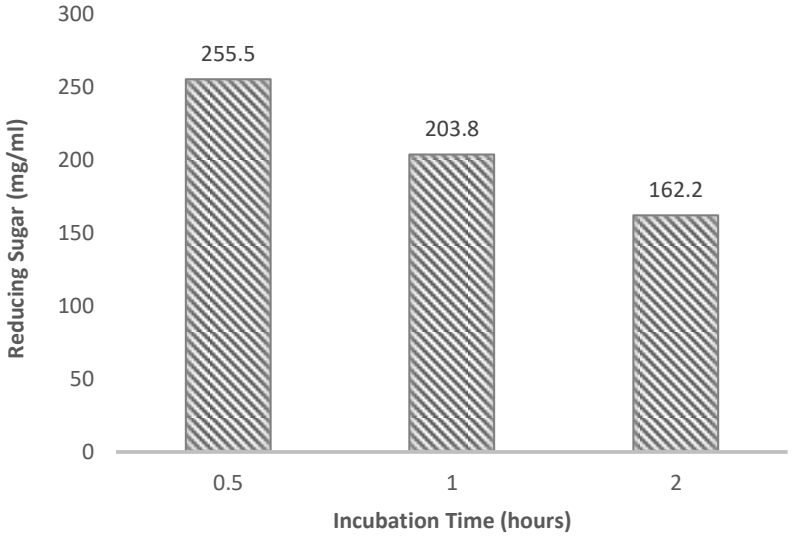

Figure 2. Reducing sugar rate on enzymatic modified of seaweed flour

Figure 2 has shown the decreasing of reducing sugar levels along with increasing incubation time. Declining levels of reducing sugar can occur due to a decrease in the rate of hydrolysis due to inactivation of cellulase absorbed in the fibrils layer on cellulose [28,29]. In addition, enzymatic hydrolysis enzyme products such as celobiose and glucose can be strong inhibitors due to enzyme inactivation over time [29]. Furthermore, the highest reducing sugar content shown in Figure 1. Was shown in seaweed flour that have been hydrolized using enzyme with a duration of 0.5 hours incubation with amount of 255.5 mg/ml. The reducing sugar produced in enzymatic modification could be used as an indicator that there has been degradation in the outer layer of seaweed cell wall containing cellulose fiber [11]. The occurrence of degradation in the outer layer of the seaweed cell wall can facilitate the release of carrageen polymers located on the inner cell wall layer [15,30].

Seaweed Kappaphycus alvarezii cultivated farmers in Potteran Island, Sumenep, Madura 45 days old contain carrageen with an average value of 76.27% of dry weight. This is in accordance with the statement of [31] which states that carrageen is one of the main constituents of red algae cell wall representing 30-75% of its dry weight.

3.4 FTIR Analysis

The characteristics of FTIR functioning groups spectra of seaweed *Kappaphycus alvarezii* before and after enzymatic pretreatment with different incubation periods (0.5 hours, 1 hour and 2 hours) are shown in Figure 3.

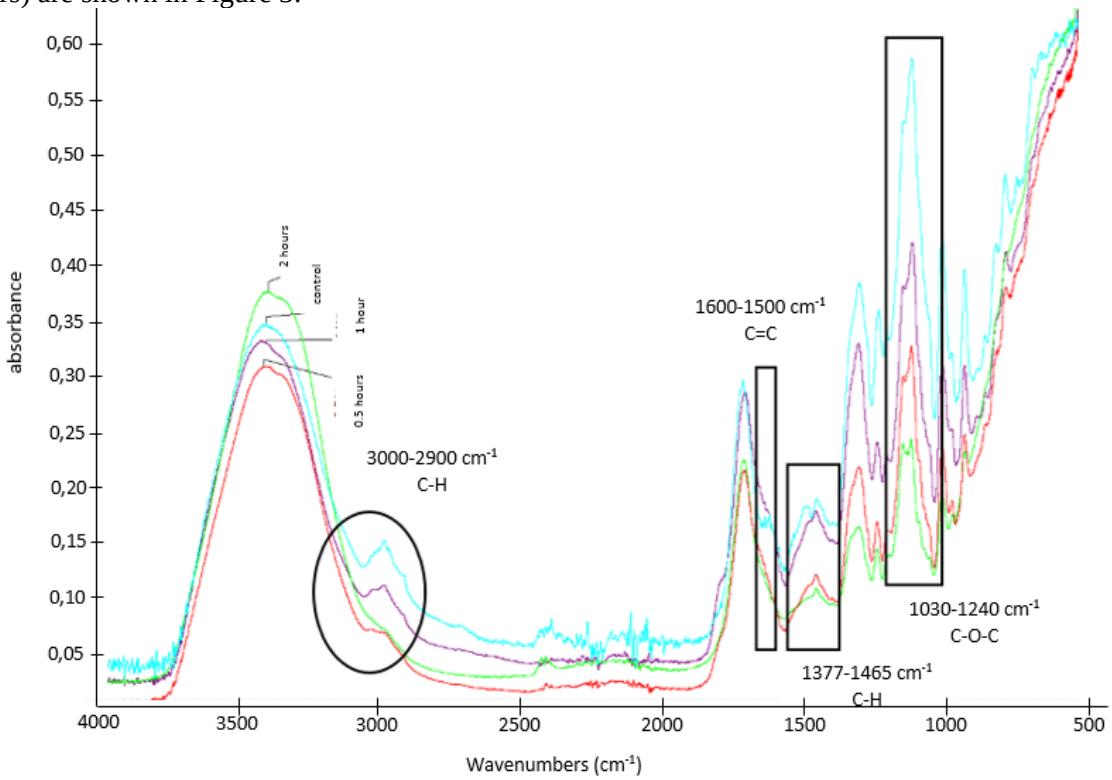

Figure 3. FTIR analysis of Seaweed Flour. Control (blue); incubation 0.5 hours (red), incubation 1 hour (purple), incubation 2 hours; (green)

According to Figure 3., the absorbance range of seaweed flour in FTIR spectra at a wavelength of 4,000-500 cm^{-1}. The broad-spectral 3000-3.700 cm^{-1} absorbance range shows the stretch group (O-H) vibration contained in seaweed flour. The absorption of clusters (O-H) on seaweed flour before being hydrolyzed enzymatically has a lower intensity at wavelengths 3,343.47 cm^{-1}.

The absorbance range of 2,900-3,000 cm^{-1} shows the stretch group (C-H) vibration characterized by peak loss in the absorbance range initially apparent in seaweed flour prior to enzymatic hydrolysis of 2,921,52 cm^{-1}. This indicates that there has been a change in chemical structure of cellulose due to the breaks in the methyl and methylene groups [32].

In addition, the loss of peak in seaweed flour resulted from enzymatic hydrolysis also appears in the absorption bands of 1,500-1,600 cm^{-1} showing the group bonding vibration (C = C) initially apparent in controlled seaweed meal (without pretreatment)

The absorption band in the range of 1,380-1,600 cm^{-1} shows the group bonding vibration (C-H). The uptake of the cluster (C-H) group on seaweed flour prior to enzymatically pretreatment shows lower intensity at wavelength 1,374.86 cm^{-1}. While on seaweed flour after enzymatic pretreatment at the incubation time of 0.5 hours and 1 hour showed higher absorption intensity that is at the wavelength of 1,375,85 cm^{-1} and 1,376,10 cm^{-1} but disappeared on modified seaweed meal with length of time 2 hours. Peak changes in the wavelength range indicate that there has been a breakdown of methyl and methylene from cellulose due to enzymatic hydrolysis [32].

The absorption band in the range 1.040-1.100 cm^{-1} shows the stretch vibration of the cluster (C-O-C). The stretch cluster (C-O-C) absorption in seaweed flour prior to enzymatic pretreatment showed lower intensity, ie, at wavelengths of 1,033.72 cm^{-1}. While on seaweed flour (using enzymatically pretreatment) with long incubation time of 0.5 hour and 1 hour showed peak shift with lower absorption intensity, that is at wavelength of 1,035,21 cm^{-1} and 1,031,87 cm^{-1} while in seaweed

flour with long 2 hours incubation time shows a peak shift with a higher absorption intensity of 1,036.19 cm^{-1}. The change in the intensity of the uptake occurs because enzymatic pretreatment using cellulase can lead to disconnection of the ether (C-O-C) group in the cellulose polymer chain [33,34].

4. Conclusions

Enzymatically pretreatment in *Kappaphycus alvarezii* flour showed that the reducing sugar decreased in content of 0.5 mg /ml after enzymatic modification at 0.5 hours incubation time. The FTIR spectra showed shifting at several functional groups namely C-H, C-O-C and C=C. Kappaphycus alvarezii contained a number of carrageenan up to 76 %, which can be the promising natural biopolymer used for fabricating hydrogels as wound dressing.

5. Acknowledgement

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