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Research Article

# Microwave Irradiation-Assisted Chitosan Hydrolysis Using Cellulase Enzyme

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**Abstract**

The influence of microwave irradiation on the chitosan hydrolysis catalyzed by cellulase enzyme was studied. The hydrolyzed chitosan was characterized by measuring its viscosity and reducing sugar. Further, it was also characterized by Fourier-Transform Infrared Spectroscopy (FT-IR), X-ray Diffraction (XRD), and Scanning Electron Microscope (SEM). The classical Michaelis-Menten kinetic parameters were measured by analyzing the amount of reducing sugars. The results were compared with the hydrolysis by using conventional shaker incubator. The hydrolysis reaction time needed to obtain similar reducing sugar yield was significantly lower for microwave irradiation than shaker incubator. On the other hand, the reduction rate of the relative viscosity was significantly higher for the hydrolysis of chitosan using shaker incubator. A significant difference in chemical structure was observed between hydrolysis using microwave irradiation and shaker incubator. Overall, the result showed that the hydrolysis behavior of chitosan using microwave irradiation is significantly different with using shaker incubator. 

***Keywords***: Microwave Irradiation; Hydrolysis; Chitosan; Cellulase

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## 1. Introduction

Chitosan is a natural polysaccharide composed of D-glucosamine and N-acetyl-D-glucosamine with $\beta$ (1-4) linkage. Unlike most polysaccharides, the presence of free electron pair of the amine group, which acts as a cation allows chitosan for binding to compounds that are anionic by an ionic or a hydrogen bond [1,2]. Chitosan has the properties of biocompatibility, degradable, bioactivity, and non-toxicity. Low molecular weight chitosan (LMWC) has been widely used in many fields, such as food [3], agriculture [4], biomaterials [5], and pharmaceutical [6,7] industries. Chitosan is synthesized by deacetylation of the chitin. In general, chitosan has high molecular weight, causing poor solubility at neutral pH and high viscosity aqueous solutions. This property inhibits wider application of chitosan.

Low molecular weight chitosan (LMWC) has been prepared by a variety of techniques, including acid hydrolysis, oxidative degradation,

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and enzymatic methods. The high acid concentration is used in acid hydrolysis of chitosan, which requires special equipment with high corrosion resistance and brings major waste disposal problems [8]. The oxidative degradation may cause some changes in its chemical structure [9]. The enzyme hydrolysis receives more attention for its mild reaction conditions, more specific, and allows a control of the extent of reaction [10]. However, enzymatic hydrolysis has a very low reaction rate. As reported by Roncal *et al*. [10], Cabrera *et al*. [11], and Lee *et al*. [12], that the chitosan enzymatic hydrolysis incubator required more reaction time.

In recent years, the microwave irradiation as a non-conventional energy source has received increasing attention in organic chemistry because of its ability to improve the selectivity, accelerate of reaction, and increase product yield compared to conventional heating [13,14]. Li *et al*. [15] studied oxidative degradation of chitosan by $H_2O_2$ under microwave irradiation. They found that the rate of degradation increased more than five times as compared to conventional heating without microwave irradiation. Saxena *et al*. [16] have performed hydrolysis of triolein under microwave irradiation. The enzymatic reaction rate of triolein was increased. Thus, the microwave is proven to increase the reaction rate and enzyme resistant to a microwave irradiation. To the best of our knowledge study of microwave-assisted chitosan enzymatic hydrolysis has not been conducted in previous publications.

In this study, chitosan hydrolysis using cellulase enzyme under a microwave irradiation was investigated to increase the reaction rate of the hydrolysis. Therefore, the research aims to study the effect of microwave irradiation on the behavior of chitosan hydrolysis using cellulase enzyme. Furthermore, comparison of microwave irradiation-assisted chitosan hydrolysis and shaker incubator-assisted chitosan hydrolysis was performed.

## 2. Materials and Methods

### 2.1 Materials

Raw chitosan was purchased from PT. Biotech Surindo, Cirebon, Indonesia. The characterization of this raw chitosan showed that the viscosity was 375.5 cps (1 % chitosan solution in 1 % acetic acid solution) and degree of deacetylation (DD) was 85.78. The protein, water, and ash contents were 0.5 %, 5.1 %, and 1.2 %, respectively. The cellulase enzyme was purchased from Sigma-Aldrich Germany (Cat. 22178) and its average activity was ~0.8 U/mg. Glacial acetic acid ($CH_3COOH$) and sodium carbonate ($Na_2CO_3$) were purchased from Merck KGaA Germany. Potassium ferricyanide ($K_3(Fe(CN)_6)$) was purchased from Sigma-Aldrich, Germany.

### 2.2 Hydrolysis of Chitosan

Chitosan was dissolved in acetic acid/ sodium acetate buffer (0.1 M) at concentration of 0.5 % and 1 % (w/v). All degradation studies were performed in a 100 mL Erlenmeyer flask. Chitosan solution (50 mL) was treated with cellulase in the ratio of 100:1 (w/w). The hydrolysis using conventional shaker incubator was carried out at the optimum process of 50 $^oC$ [17] and 100 rpm. Samples were withdrawn at every 15 min during the first hour. Then, the next samples were withdrawn at every 30 min up to a 3$^{rd}$ hour and every 1 hour up to 8$^{th}$ hour. The last sample was withdrawn after 24 hours.

The procedure for chitosan hydrolysis using microwave irradiation was almost similar with shaker incubation. The hydrolysis using microwave irradiation was carried out with the power of 40 and 120 watts. Samples were withdrawn from the first minute up to 10 min. The reaction was stopped by heat inactivation of the enzyme in a boiling water bath for 10 min. Then the samples were subjected to viscosity and reducing sugars analysis. A part of samples was adjusted to pH 8.0 with 10 % NaOH solution, resulting in a precipitate, and oven dried prior to FTIR, XRD, and SEM analysis.

### 2.3 Analysis

Relative viscosity was measured as the decrease in viscosity of the chitosan solution using Ostwald viscometer at 28±1 $^oC$ and determined using Equation (1) [10].

$$\eta_{rel} = t / t_0 \qquad (1)$$

where, $\eta_{rel}$ is relative viscosity, $t$ is the flow time of chitosan sample solution, and $t_0$ is the flow time of initial chitosan solution in the absence of hydrolysis.

Analysis of total reducing sugars was conducted according to Imoto method [9]. The Imoto reagent was prepared as follows: 0.50 g of potassium ferricyanide was mixed with 1 L of 0.5 M sodium carbonate solution and then was stored in a glass bottle protected from light. A mixture containing 4 mL of Imoto reagent and 3 mL of sample was heated for 15 min at 100 $^oC$ followed by cooling to room temperature.

The absorbance of the mixture solution was measured at 420 nm. Total reducing sugars were calculated based on a standard curve obtained with D-glucosamine HCl.

FTIR spectra were recorded with KBr powder using the instrument IR Prestige-21 Shimadzu, Japan. Thirty-two scans were performed at a resolution of 4 $cm^{-1}$ and a temperature of 21±1 $^oC$ over the wavelength range of 500-4,000 $cm^{-1}$. XRD patterns were obtained by a Shimadzu Lab XRD -7,000 diffractometer and use a CuKα target at 30 kV and 30 mA at 20 $^oC$. The scattering range (2θ) was recorded from 5 to 400. The relative crystallinity (RC) of chitosan was calculated using Equation (2) [18].

$$RC\ (\%) = (Ac/(Ac + Aa))*100 \quad (2)$$

where, *Ac* is the crystalline area and *Aa* is the amorphous area. The morphology of chitosan was examined using a scanning electron microscope (JEOL JSM-6510LA SEM, Japan) at an acceleration voltage of 10 kV. The sample was coated with platinum and a magnification of 10,000 X.

## 3. Results and Discussion

### 3.1 The Effect of Hydrolysis Reaction on Viscosity and Reducing Sugar

The change in the viscosity of the linear polysaccharide solution by enzymatic degradation reflects the change in the degree of polymerization [11]. Thus, the course of degradation of chitosan samples was conveniently studied by viscosity measurement, and characterized by the decrease of the relative viscosity at a given time of degradation [11,19]. The relative viscosity as a function of incubation time on chitosan sample concentration of 0.5 % and 1 % (w/v) under the shaker incubator are shown in Figure 1A.

The relative viscosity of the reaction mixture dropped dramatically in the early reaction stage. The hydrolysis of the initially long chitosan chains caused greater viscosity decreases than the subsequent degradation of their shorter hydrolysis products. Fast degradation during the initial hydrolysis stage is attributed to endo-enzyme activity [20]. After 4 h, degradation slowed down. This phenomenon is probably due to the inhibition of the enzyme activity by the end products [19].

Each enzymatic cleavage of relatively rigid linear polysaccharides generates the new reducing sugar. The formation of reducing sugar indicates the activity of an enzyme that cleaves glycosidic bond in chitosan polymer. The effect of hydrolysis reaction time on reducing sugar formation are shown in Figure 1B. It shows that within the first hour, a relatively fast formation of reducing sugar was observed. After that, decreasing their formation rates in the following 4 h was observed. The formation of reducing sugar of chitosan samples seemed to be constant after 8 h of hydrolysis.

Chitosan degradation in 0.1 M acetic buffer solution with cellulase enzyme under microwave irradiation was investigated. Figure 2A shows that the rate of the relative viscosity decrease is significantly lower compared with shaker incubator. Then, the formation rate of reducing sugar is significantly higher for microwave irradiation (Figure 2B). Under the condition of microwave irradiation, the yield

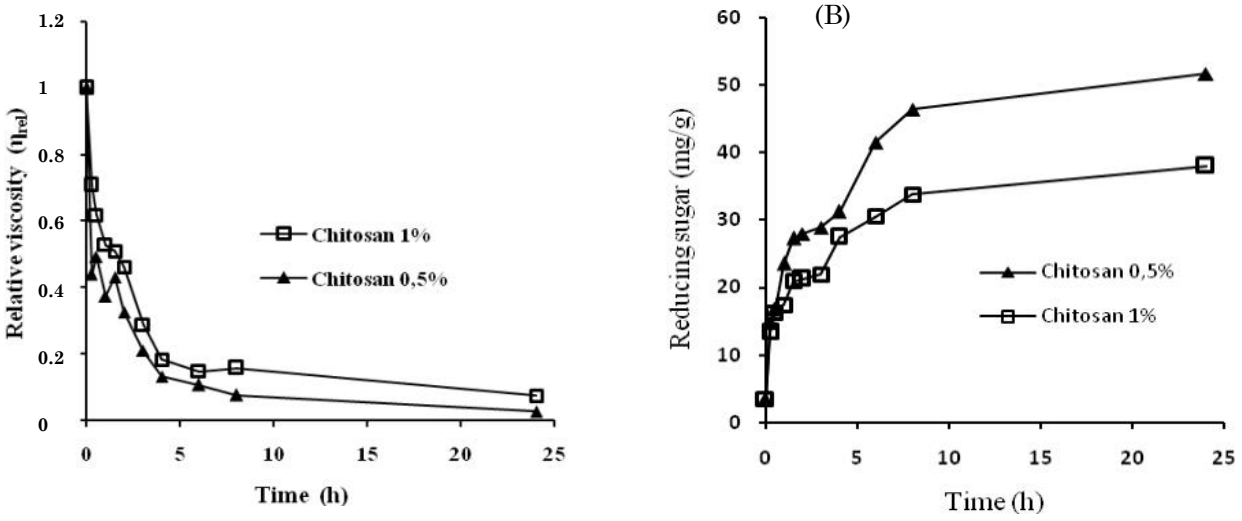


**Figure 1.** The effect of reaction time at chitosan hydrolysis using shaker incubator (temperature 50 $^oC$, 100 rpm) on relative viscosity (A) and reducing sugar (B)

reducing sugar is approximately 25 mg/g after 10 min hydrolysis. By contrast, to obtain similar yield (25 mg/g), it is required 3 hour for hydrolysis using shaker incubator (Figure 1B). It can be explained that microwave irradiation induces polar molecules. The oscillating electric field from microwaves creates friction among molecules resulting in fast heating [21]. In addition, although the microwave irradiation only contains one joule per mole of photons, it can reduce activation of the Gibbs energy of reaction through microwave energy storage in the vibrational energy via effect enthalpy and entropy of molecule [22]. Consequently, the activation reaction in a polar bond such as glycoside bond increased and the cleavage of glycosidic bond occurs on the reducing end of chitosan [15]. Thus, this phenomenon causes the reduction rate of the relative viscosity decreased and the formation rate of reducing sugars increased. This is in accordance with the characteristics of the exo-type reaction that has been described by Lee *et al*. [12] and Li *et al*. [19].

The degradation of chitosan under microwave irradiation with different power was studied. The effect of hydrolysis reaction time at different power on relative viscosity and reducing sugar are shown in Figure 3. The relative viscosity decreased (Figure 3A) and the reducing sugar formation increased (Figure 3B) with increasing power. This phenomenon can

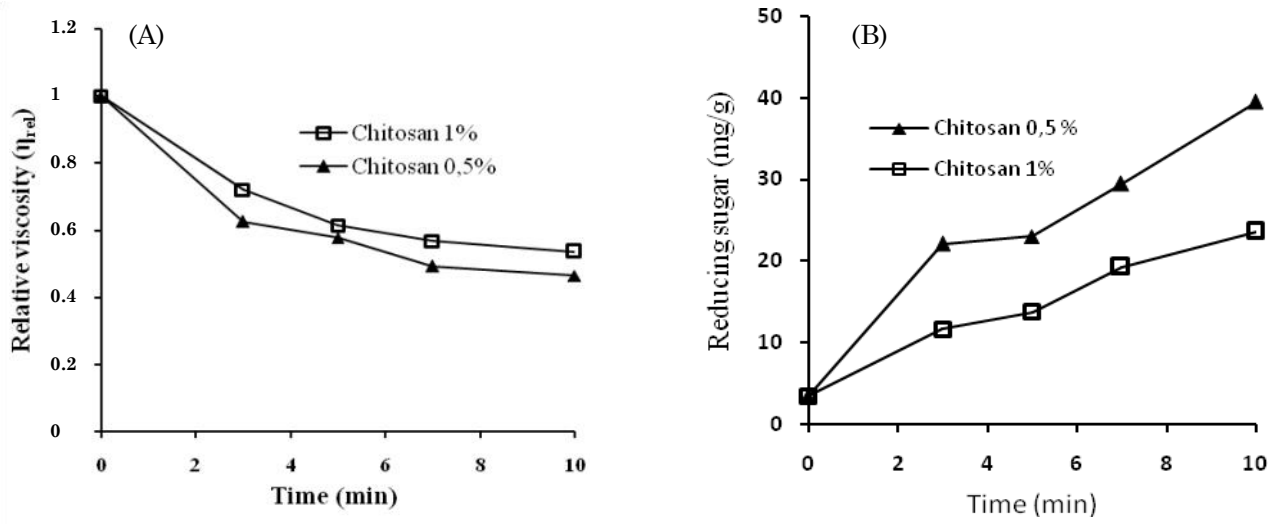


**Figure 2.** The effect of reaction time at chitosan hydrolysis using microwave irradiation (power 40 watt) on relative viscosity (A) and reducing sugar (B)

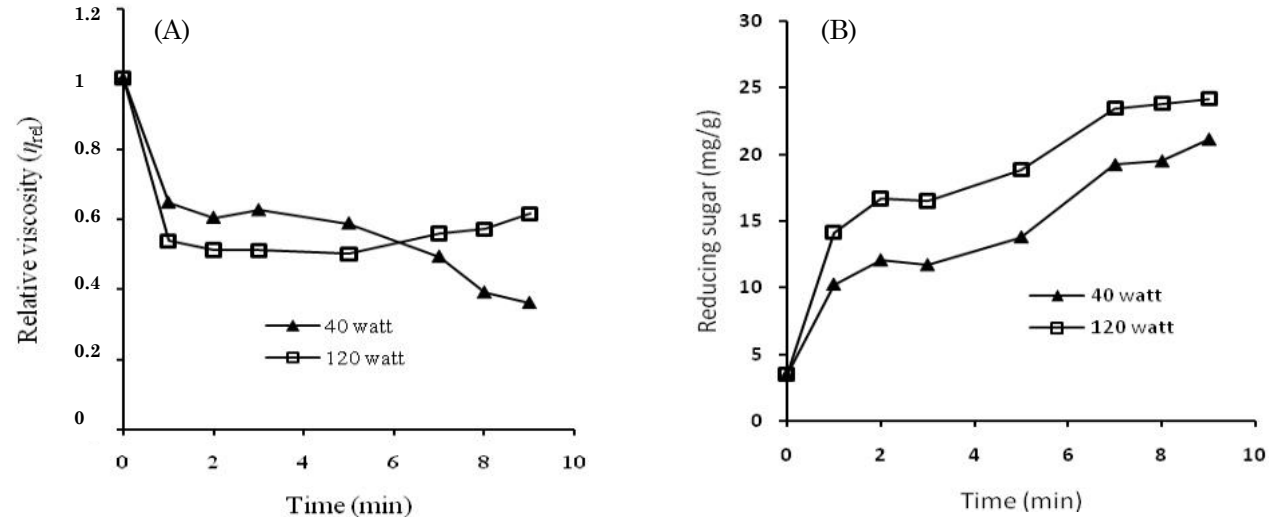


**Figure 3.** The effect of hydrolysis reaction time at different microwave power on relative viscosity (A) and reducing sugar (B)

be explained that increasing power can increase the resulting photons thus the cleavage rate of a glycosidic bond increased. This statement is well explained by Mello [21]. However, the relative viscosity increased after 5 minutes at a power of 120 watts (Figure 3A). This increase can be explained that the heating rate of material increases with increasing microwave power. Because the solvent is easily evaporated, therefore the viscosity increased.

The reducing sugar yield of degraded chitosan products increased with increasing power. The degraded products with similar reducing sugar yield required shorter reaction time at high power level than at low power level (Figure 3B). Under a microwave irradiation field, the intensified molecular motions and collisions enable a high probability of contact between substrate and enzyme, resulting in an enhanced reaction rate with increasing power. This statement similar to the previously publication by Li *et al*. [15] and Su *et al*. [23].

3.2 Kinetics of the Chitosan Hydrolysis

Michaelis-Menten kinetic parameters (Equation (3)) were measured by analyzing the amount of reducing sugars. Kinetics of enzymatic hydrolysis of chitosan were investigated using varying initial concentration of the chitosan with using shaker incubator (at 50 °C and 100 rpm), and microwave irradiation (at power of 40 watt).

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \tag{3}$$

where, $V$ is the reaction rate, $V_{max}$ is the maximum reaction rate, $K_m$ is the Michaelis–Menten constant, and [$S$] is substrate concentration.

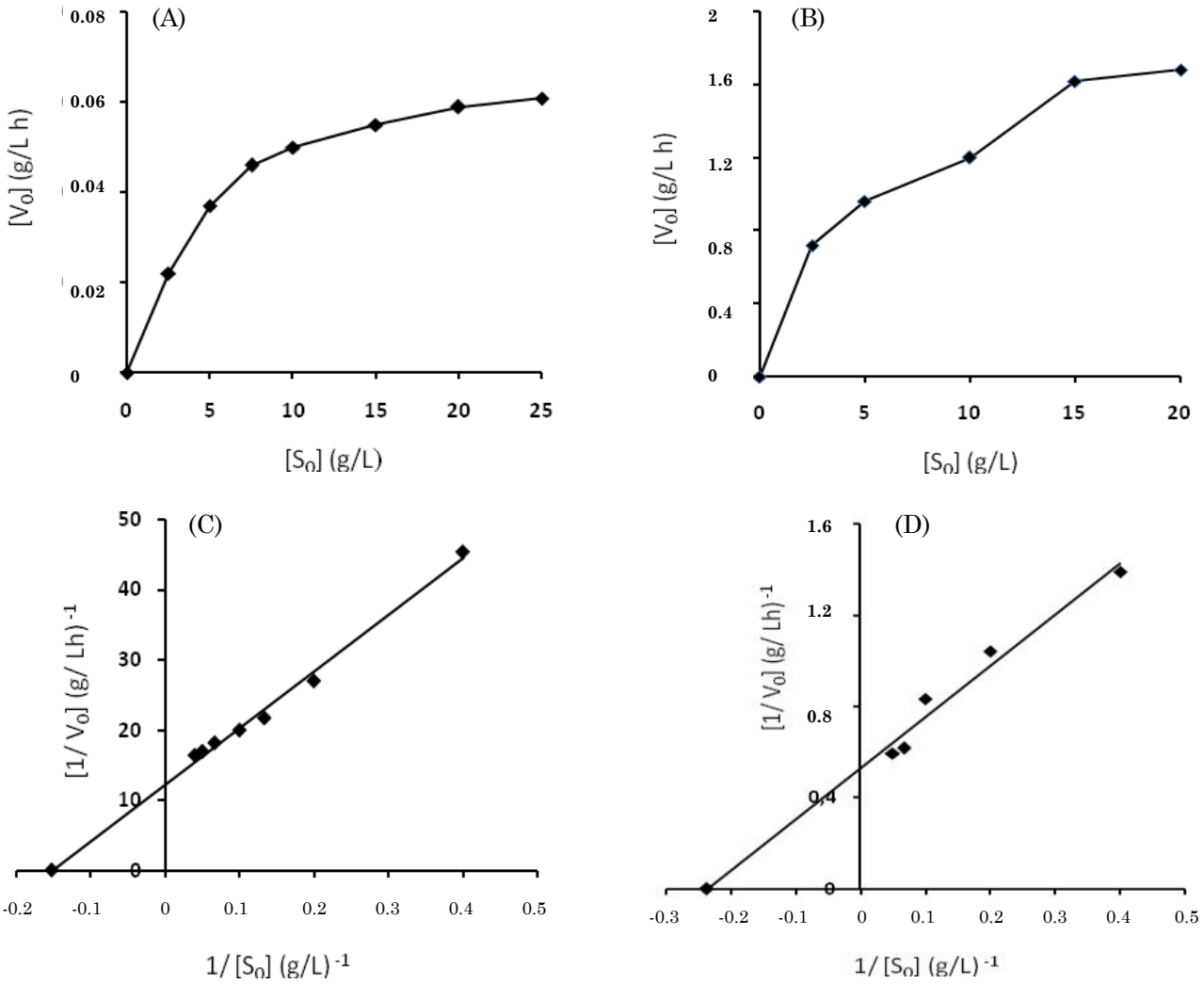


**Figure 4.** The effect of substrat concentration on initial velocity of reducing sugar formation at chitosan hydrolysis: (A) using shaker incubator, (B) using microwave irradiation. Lineweaver-Burk plots: (C) using shaker incubator, (D) using microwave irradiation

Figure 4A and 4B shows the effect of varying substrate concentration on initial degradation rates. Lineweaver burk plot (Figure 4C and 4D) showed the $V_{max}$ for irradiation microwave (1.98 g/L h) was higher than for shaker incubator (0.0814 g/L h), while $K_m$ for the irradiation microwave (4.228 g/L) was lower than for shaker incubator (6.516 g/L). $K_m$ is the measure of the strength of enzyme-substrat complex. A higher $K_m$ indicates weak binding, whereas the lower value indicates strong binding [19].

3.3 The Properties of Degraded Chitosan

The chemical structure of raw and hydrolyzed chitosan was analyzed by FTIR spectroscopy. The spectrum of raw chitosan (Figure 5A) shows the –CH vibration band at wavelength of 2,900 $cm^{-1}$, $CH_2$ bond at wavelength of 1,423 $cm^{-1}$, and $–CH_3$ bond at wavelength of 1,379 $cm^{-1}$. Furthermore, the absorption band at wavelength of 1,153 $cm^{-1}$ indicates –C–O–C– glycosidic linkage between chitosan monomer. These results are in agreement with previous publications by Kumar *et al*. [24] and Prasertsung *et al*. [25].

In both hydrolyzed chitosan using shaker incubator (Figure 5B) and using microwave irradiation (Figure 5C) are not observed additional peak, but the peak intensity at wavelength of 2,900 $cm^{-1}$ and 1,153 $cm^{-1}$ decreases. The peak intensity at a wavelength of 1,153 $cm^{-1}$ for hydrolyzed using microwave irradiation is lower than using shaker incubator. This indicated that more the cleavage of the glycosidic linkage for hydrolyzed using microwave. In hydrolyzed chitosan using microwave irradiation, the bands at wavelength of 1,410 $cm^{-1}$ (symmetrical deformation of $CH_3$ and $CH_2$) and 1,370 $cm^{-1}$ (bending and stretching of $CH_3$ and $CH_2$) are weak. This weakening in the absorption peak for hydrolyzed using microwave irradiation indicates a hydrogen bonding decrease in the inter-molecular and intra-molecular. This statement is similar with the previous results described by Kumar *et al*. [24].

The X-ray diffraction pattern of the raw chitosan, the hydrolyzed chitosan using shaker incubator, and the hydrolyzed chitosan using microwave irradiation are shown in Figure 6. The raw chitosan displayed the characteristic peaks at 2θ = 20.40, 29.30, and 340. Compare with raw chitosan, the peak intensity of hydrolyzed chitosan at 2θ = 20.4 increases while at 2θ = 29.3 decreases. Hydrolysis of chitosan led to the relative crystallinity decrease. The relative crystallinity of hydrolyzed chitosan using microwave irradiation is lower than using shaker incubator. According to Li *et al*. [26], there is a correlation between depolymerization and crystallinity. FTIR characterization showed that there was a hydrogen bonding decrease in the inter-molecular and intra-molecular for hydrolyzed using the microwave. This is evidenced by the larger amorphous area of chitosan crystals for the hydrolyzed using microwave irradiation. Thus the crystallinity of hydrolyzed chitosan using microwave decreased causing easier to be degraded and dissolved.

Scanning electron microscope was used to visualize the morphology of raw chitosan and

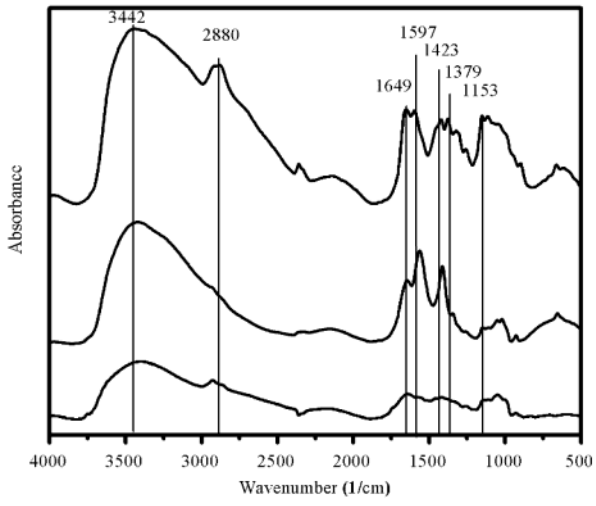


**Figure 5.** FTIR spectra of chitosan: (A) Raw chitosan. (B) Hydrolyzed chitosan using shaker incubator. (C) Hydrolyzed chitosan using microwave irradiation.

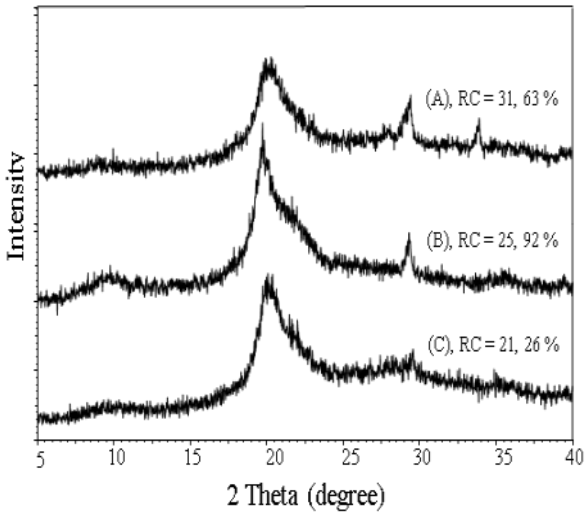


**Figure 6.** X-ray diffraction patterns of chitosan: (A) Raw chitosan. (B) Hydrolyzed chitosan using shaker incubator. (C) Hydrolyzed chitosan using microwave irradiation.

hydrolyzed chitosan. The SEM results of the raw chitosan (Figure 7A) shows a non-porous, smooth, microfibrils, and crystallites, which was similar with observation by Singh *et al*. [27] and Lou *et al*. [28]. After treatment, some coarseness or pits has been generated on the surface of chitosan. The morphological differences between hydrolyzed chitosan using shaker incubator (Figure 7B) and using microwave irradiation (Figure 7C) can be observed. In the SEM results of the hydrolyzed chitosan using shaker incubator, some erosion of amorphous material from the surface of the fibers can be detected. It is relevant to the SEM result of degradation of chitosan by pulsed electric fields treatment [28]. While the SEM results of the hydrolyzed chitosan using microwave irradiation shows clear edges of the wall structure of the chitosan molecule which was broken. This indicated that the cleavage of glycosidic bond for the hydrolyzed of chitosan using microwave irradiation should occur at the end of the chitosan chain.

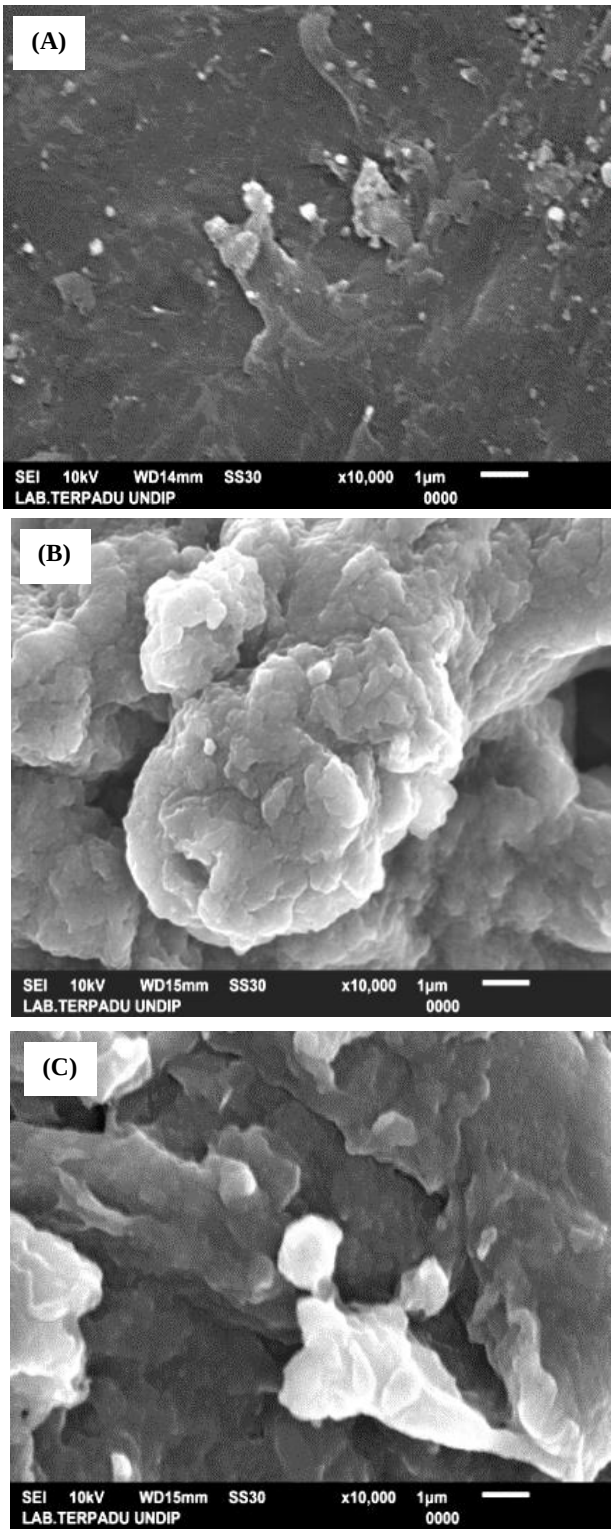


**Figure 7.** SEM of chitosan: (A) Raw chitosan. (B) Hydrolyze chitosan using shaker incubator. (C) Hydrolyze chitosan using microwave irradiation

## 4. Conclusions

In the current study, the enzymatic hydrolysis of chitosan by using microwave irradiation was investigated and then compared with using conventional shaker incubator. The results indicated that microwave irradiation accelerates the cleavage rate of chitosan chain. The required reaction time to results in the similar reducing sugar for microwave irradiation is shorter than shaker incubator. The reducing sugar yield increases with increasing microwave power. The viscosity measurements showed that the reduction rate of the specific viscosity for the chitosan hydrolysis using shaker incubator is higher than microwave irradiation. The Michaelis-Menten kinetics showed $V_{max}$ of 1.98 mg/mL.h and $K_m$ of 4.228 mg/mL for hydrolysis using the microwave irradiation, whereas $V_{max}$ of 0.0814 mg/mL.h and $K_m$ of 6.516 mg/mL for hydrolysis using the shaker incubator. Considering the hydrolysis location, the chemical structure of hydrolyzed chitosan using microwave irradiation should be better than hydrolyzed chitosan using shaker incubator (the end of chitosan chain vs random cutting chain chitosan).

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