Characterization of aquatic plant (Egeria densa) pre-treated by alkaline hydrogen peroxide

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Abstract. Egeria densa is an aquatic plant growing very rapidly in freshwater. It is very potential as a lignocellulose source to produce bioethanol. However, the challenge of bioethanol production from lignocellulose biomass is the difficulty of hydrolyzing lignocellulose because cellulose in biomass is blocked by lignin and hemicellulose, and it has high crystallinity. To overcome the problems, pre-treatment process is absolutely required. The objectives of this study were to characterize the chemical compound of Egeria densa and to evaluate the effect of concentration, temperature and time pretreatments of alkaline hydrogen peroxide (AHP) on yield, physical and chemical structures of Egeria densa. Dried biomass of Egeria densa contained 35.47\pm0.77\% of hemicellulose, 13.56\pm0.61\% of cellulose, and 13.58\pm0.22\%; of lignin. The yield and lignin content of pretreated E. densa decreased with increasing H\textsubscript{2}O\textsubscript{2} concentration, temperature and time of pretreatment. In addition, the structure of pretreated E. densa became smoother compared with its untreated. The structure of cellulose was changed after pre-treatment, from the crystalline to more amorphouse form. Alkaline hydrogen peroxide pre-treatment also could produce simple sugar, such as glucose, maltose and arabinose, which important for bioethanol conversion.

Keywords: bioethanol, Egeria densa, lignocellulose, pre-treatment

1. Introduction
Bioethanol is an alternative biofuel to substitute or replace gasoline because it has similar physical and chemical properties (Naik et al 2010). Currently, Brazil and the United States are the main producers of bioethanol with the share contribution about 85\% of total annual bioethanol production (RFA 2018). However, they use food material material such us sugar crops, starch corps, that may threaten food security. Regarding this problem, it is crucial to find a new feedstock resources which is high sustainability and availability. Lignocellulose biomass has potential as a new feedstock resources for bioethanol production due to it has several advantages such as widely-distributed, cheap, and not compete with food material. In recent years, researchers has used terrestrial biomass to produce bioethanol, such as soft wood, hard wood, herbaceous and agricultural crops waste (McKendry 2002, Lynd et al 2008).
Egeria densa is a freshwater aquatic plant, which has potential as a lignocellulose biomass for bioethanol production. *E. densa* can grow very rapidly with the productivity 497 g dry weight/m²/month (Tanimizu and Miura 1976). *E. densa* can replace the vegetation of native aquatic plants in a water, disrupt ship navigation, clog waterways, trap sediments, and inhibit migration of anadromous fish (Foschi *et al* 2004). Utilization of *E. densa* is only limited as an aquarium ornamental plant, therefore it is very potential to be developed into bioethanol as an effort to overcome the uncontrolled growth of *E. densa*.

Lignocellulose biomass contains cellulose and hemicellulose which can be converted to bioethanol by saccharification and fermentation process (Balat 2008). The difficulty of producing bioethanol from lignocellulosic biomass is to hydrolyze lignocellulose because cellulose in lignocellulose is blocked by lignin and hemicellulose, thus inhibiting the function of cellulase enzymes. Cellulase also has high crystallinity due to the inter and intramolecular hydrogen bonds which make cellulose insoluble and difficult to hydrolyze (Puri 1984). To overcome this problem, pretreatment of lignocellulose before the saccharification is absolutely needed. The pretreatment aims to enhance the accessibility of cellulase enzymes in cellulose by removing lignin and hemicellulose (Chang and Holtzapple 2000) and decreasing the crystallinity of cellulose (Yosida *et al* 2008). Steam, liquid acid, alkaline and oxidative pretreatment methods have been widely used for lignocellulosic pretreatment. Oxidizing compounds in oxidative pretreatments such as hydrogen peroxide are added to biomass (Hendriks and Zeeman 2009). Alkaline peroxide pretreatment is more effective than alkaline pretreatment in dissolving lignin (Chen *et al* 2008) and increasing residual digestibility (Talebnia *et al* 2010).

Alkaline Hydrogen Peroxide (AHP) pretreatment for lignin dissolution depends on the pH value of the solution. Gould (1985) showed that pH 11.5-11.6 is the optimum pKa for the H₂O₂ dissociation reaction. Lignin oxidation of species in this reaction is highly reactive to hydroxyl radicals (HO⋅) that are formed during H₂O₂ degradation in reactions with hydroperoxy anions (HOO⋅) (Lewis *et al* 1987). The great advantage of AHP pretreatment is not leaving any residue in the biomass because of degradation to oxygen and water and can also be done at room temperature (Rabelo *et al* 2008). Alkaline hydrogen peroxide pretreatment is expected to streamline the delignification process and decreases the crystallinity of cellulose from *E. densa*. The objective of this study was to characterize the chemical compound of *E. densa* and to evaluate the effect of concentration, temperature and time pretreatments of alkaline hydrogen peroxide on yield, physical and chemical structures of *E. densa*. This research can provide information on the potential of *E. densa* to be used as feedstock for bioethanol with using alkali hydrogen peroxide as an effective pretreatment agent.

2. Materials and methods

2.1. Materials

*E. densa* was collected from Gunung Bunder, Kabupaten Bogor, West Java. Hydrogen peroxide dan sodium hydroxide were purchased from PT Bratacem, Bogor.

2.2. Methods

2.2.1. Chemical composition of *E. densa* biomass. Schematic of the experimental procedure are shown in figure 1. *E. densa* biomass was briefly dried by sunlight for a day, then ground and screened using 37 mesh screen. The chemical composition of dry biomass, such as hemicellulose, cellulose and lignin were analyzed using the Goering and Van Soest (1970) method.
2.2.2. Pretreatment of *E. densa*. The pretreatment of dried biomass of *E. densa* referred to Rabelo *et al* (2011), in which alkaline hydrogen peroxide, time and temperature were modified. Briefly, the hydrogen peroxide solution was adjusted to pH 11.5 using NaOH 6 M (Coreia *et al* 2013). The dried biomass (500 mg) was mixed with 10 mL of alkaline hydrogen peroxide with concentration 2 or 10%. The biomass was heated 30 or 60°C for 1 or 6 h and time of pretreatment 1 or 6 h (figure 1). Then, the samples were filtered to obtain regenerated water (RW) and regenerated biomass.

The lignin content extracted in the RW was examined by UV-VIS Spectrophotometer RS at a wavelength of 283 nm (Uju *et al* 2013). Regenerated biomass was dried and weighed to determine the percentage of Regenerated Biomass (YRB). Yield Regenerated Biomass (YRB) is calculated based on the following equation:

\[
\text{YRB (\%)} = \frac{\text{Mass of dried regenerated } E.\ densa}{\text{Mass of dried untreated } E.\ densa} \times 100\%
\]

2.2.3. Scanning Electron Microscopy (SEM) analysis. The morphology of dried untreated and regenerated of *E. densa* were observed using Scanning Electron Microscopy (SEM). The sample on a metal-cylinder plate was coated with gold before analysis. SEM analysis was performed using Zeiss Evo MA-10, and SEM imgaged was recorded at 250 and 10,000 magnifications.

2.2.4. Fourier transform infrared (FT-IR) spectroscopic analysis. The aim of FT-IR analysis was to characterize the cellulose and lignin structure on the dried untreated and regenerated biomass of *E. densa*. The sample was mixed with KBr and pressed to form a thin film. The infrared spectrum was recorded using a Bruker Tensor 37 fourier transform infrared spectrophotometer for the over range 1800-800 cm⁻¹ with four scans recorded at 4 cm⁻¹ resolutions.

2.2.5. X-ray diffraction (XRD) analysis. XRD analysis was performed using XRD Bruker D8 Advance to characterize the crystalinity of the dried untreated and regenerated *E. densa*. The sample was scanned over a diffraction angle (2θ) from 5 to 50° at a step size of 0.02°.

![Figure 1. Schematic of the experimental procedure.](image-url)
2.2.6. **Reducing sugar analysis.** The reducing sugar of regenerated water was analyzed using using dinitrosalicylic colorimetric method (Miller 1991). To estimate the reducing sugar on the regenerated water of *E. densa*, the glucose was used to make a standard curve. The regenerated water or the glucose solution was mixed with the dinitrosalicylic acid reagent solution and heated on the boiled water for 5 min. After heated, the reaction mixture solution was cooled to room temperature, then examined by UV-VIS Spectrophotometer at a wavelength of 540 nm.

2.2.7. **Profiling sugar content.** Profiling saccharide content in the RW was determined by a High Performance Liquid Chromatography (HPLC) with Aminex-HPX 87H column. The deionized water containing 0.04% trifluoroacetate (v/v) was used as a mobile phase. A 20 µL of regenerated water was injected, then recorded using Reflective Index Detector for 15 minutes. The standard sugars used in this analysis are xylose, mannose, maltose, glucose, arabinose, and galactose.

### 3. Results and discussion

3.1. **Chemical composition of *E. densa***

The chemical composition of *E. densa* biomass was presented in table 1. *E. densa* biomass had high content of hemicellulose, but low content of cellulose and lignin. The hemicellulose content of *E. densa* was higher than the aquatic plant *Eichhornia crassipes* (Poddar et al 1991) and terrestrial lignocellulose biomass (McKendry 2002). The hemicellulose and cellulose are a major component of lignocellulose biomass, which can be used for bioethanol production. Hemicellulose is a natural polymer composed of pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose). On other hand, cellulose is a polysaccharide consisting a linear chain of glucose units linked with β-1-4-glycosidic bonds (McKendry 2002). The lignin content of *E. densa* was slightly higher than switch grass and aquatic plant *Eichhornia crassipes* (table 1). The high lignin content of biomass can inhibit the hydrolysis of hemicellulose and cellulose into saccharides, and cause the low bioethanol conversion. The efficiency of bioethanol conversion depends on the structure and composition of lignocellulose biomass.

| Samples                | Hemicellulose (%) | Cellulose (%) | Lignin (%) |
|------------------------|-------------------|---------------|------------|
| *Egeria densa*         | 35.5±0.8          | 13.6±0.6      | 13.6±0.2   |
| *Eichhornia crassipes* | 18.42             | 25.61         | 9.93       |
| Softwood              | 25-30             | 35-40         | 27-30      |
| Hardwood              | 20-25             | 45-50         | 20-25      |
| Wheat straw           | 20-25             | 33-40         | 15-20      |
| Switchgrass           | 10-40             | 30-50         | 5-20       |

a) Poddar et al (1991), and b) McKendry (2002).

3.2. **Lignin content on regenerated water***

Lignin content on the regenerated water (RW) was estimated by measuring the absorbance at 283 nm. As presented in figure 2, RWs from pretreated *E. densa* have the high absorbance value (0.89±0.05 - 1.08±0.01), indicated that the lignin was removed from dried *E. densa* biomass after pretreatment. Sun *et al* (2000) reported that the decrease in absorbance value is caused by oxidation of lignin degradation by alkali hydrogen peroxide when the reaction factor is increased.
3.3. Characteristics of pretreated E. densa biomass

3.3.1. Yield Regenerated Biomass. The dried pretreated E. densa was weighted to obtain percentage of yield regenerated biomass (YRB). The YRB range from 24.4 to 42.7% and there showed a tendency to decrease with increasing concentrations of alkaline hydrogen peroxide, temperature and time of pretreatment, as shown in figure 3. The highest YRB was found at 2% H₂O₂ at 30°C for 6 hours (42.78%), while the lowest percentage was found at a 10% H₂O₂ concentration at 60°C for 6 hours (24.39%). The low of YRB was caused by the high level of delignification and the depolymerization of hemicellulose and cellulose so that it dissolves in regenerated water. Increased pretreatment parameters such as concentration, temperature, and time are known to increase delignification which indicates that more compounds are decomposed by hydrogen peroxide (Sun et al 2000).

![Figure 2. The lignin content of regenerated water.](image)

![Figure 3. Effect of alkaline hydrogen peroxide pretreatment on YRB of E. densa.](image)
3.3.2. **SEM analysis.** SEM images of untreated and pretreated *E. densa* biomass are presented in figure S1. The structure of the regenerated biomasses (RB) became smoother than its untreated biomass. The untreated biomass has tight and regular structure, while the RB has swelling and no visible granular structure at magnification of 10,000 times. The structure changes presumably were caused by partial removal of the lignin. As reported by Martel and Gould (1990), after the pretreatment of alkaline hydrogen peroxide, the cell wall loses most of the rigid structure and the textures become non-uniform, thereby facilitating access to enzymatic hydrolysis. Swelling occurred because lignin dissolved and a portion of hemicellulose is reduced (Coreia *et al* 2013). The damaged lignocellulose structure and the increase in surface area make the biomass bioaccessibility increased (Lin *et al* 2015).

3.3.3. **FTIR analysis.** The FT-IR spectral data are shown in figure 4. For instance, peak bands were observed clearly at wavenumber 896 cm⁻¹, which is attributed to the presence of β-1,4-glycosidic linkages of the glucose ring of cellulose chain (Lamaming *et al* 2015). Additionally, the band at 1326 cm⁻¹ can attributed to the typical structure of lignin due to the syringyl ring plus guaiacyl ring condensed (El Mansouri *et al* 2011). Compared with the untreated biomass, the alkaline hydrogen peroxide pretreatment changed the structure of regenerated biomasses. The significant change was observed on the regenerated biomass pretreated with high concentration alkaline hydrogen peroxide with high temperature and longer time reaction (10% H₂O₂, 60°C for 1 h).

The band of β-1,4-glycosidic in cellulose was shifted and the intensity decreased after pretreatment, suggested that the structure of cellulose was changed. In addition, the band at 1326 cm⁻¹ was disappeared with increasing the reaction factor (concentration alkaline hydrogen peroxide, time and temperature), indicated that the lignin was removed from *E. densa* biomasses. This result agreed with the lignin content of RW (figure 2).

![Figure 4. FT-IR spectra of untreated and pretreated *E. densa* biomass.](image)

3.3.4. **XRD analysis.** XRD analysis was aimed to measure the changing of cellulose structure on *E. densa* biomass after alkaline hydrogen peroxide pretreatment. The cellulose has two chain form region, namely crystalline (cellulose I) and hydrophilic (cellulose II), which can identify from XRD diffractogram. The XRD diffractogram of the untreated and pretreated *E. densa* biomasses is shown in figure 5. The untreated *E. densa* has two strong peaks at 21.4 and 14.3°, which indicated that it had cellulose I characteristics. After the *E. densa* had been pretreated by alkaline hydrogen peroxide, the peak at 21.4 and 14.3° disappeared. This indicate that the alkaline hydrogen peroxide pretreatment could change the crystalline structure of cellulose to more amorphous cellulose fractions.
3.4. Reducing sugar and sugar profile

The reduction of sugar content was used to measure the effect of AHP pretreatment on the solubilization of cellulose on the RW. We analyzed the reducing sugar of RW from *E. densa* pretreated with lowest and highest of concentration AHP, temperature and incubation time, as shown in figure 6a. The reducing sugar content of RW from both pretreatments does not significant difference, thus suggested that the amount of solubilization of cellulose from *E. densa* is similar.

![Reducing sugar content](image)

**Figure 6.** The reducing sugar content (a) and sugar profile (b) of regenerated water. (■ = 2% 30ºC 1 jam), (□ = 10% 60ºC 6 jam).

In addition, we also analyzed the sugar profile of RW of *E. densa* pretreated with lowest and highest of
reaction factor, including concentration AHP, temperature and time pretreatment, as shown in figure 6b. The difference of reaction factor of pretreatment influenced the sugar profile. The RW of *E. densa* pretreated with lowest reaction factor contains glucose and maltose, whereas the RW from highest reaction factor contains glucose and arabinose. The glucose and maltose are the depolymerization product from cellulose, while the arabinose is the degraded product from hemicellulose. This result suggested that the AHP pretreatment with highest reaction factor could depolymerized the hemicellulose of biomass. The simple sugar, such as glucose, maltose and arabinose can be converted to bioethanol by fermentation.

4. Conclusion

*E. densa* had high potency for bioethanol feedstock. Pretreatment using alkaline hydrogen peroxide could remove lignin and hemicellulose and decrease the crystallinity of cellulose. Alkaline hydrogen peroxide pretreatment could produce simple sugar, such as glucose, maltose and arabinose, which important for bioethanol conversion.

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Figure S1. SEM images of untreated and pretreated *E.densa* biomasses

Untreated of *E. densa* biomass (250x)

Untreated of *E. densa* biomass (10,000x)

*E. densa* pretreated with 2% H₂O₂, at 30°C, 1 h (250x)

*E. densa* pretreated with 2% H₂O₂, at 30°C, 1 h (10,000x)

*E. densa* pretreated with 2% H₂O₂, at 30°C, 6 h (250x)

*E. densa* pretreated with 2% H₂O₂, at 30°C, 6 h (10,000x)
E. densa pretreated with 10%, H₂O₂, at 60°C, 1 h (250x)

E. densa pretreated with 10%, H₂O₂, at 60°C, 1 h (10,000x)

E. densa pretreated with 10%, H₂O₂, at 60°C, 6 h (250x)

E. densa pretreated with 10%, H₂O₂, at 60°C, 6 h (10,000x)