

Physical and Biophysical Pretreatment of Water Hyacinth Biomass for Cellulase Enzyme Production

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doi: 10.15255/CABEQ.2015.2284

Original scientific paper

Received: July 27, 2015

Accepted: June 9, 2016

One practical way to control water hyacinth overgrowth due to eutrophication is by utilizing it as a substrate to produce cellulase. Water hyacinth was subjected to pretreatment to degrade lignin and improve microbes' accessibility to cellulose. Physical and biophysical pretreatment methods were investigated. Biomass size reduction was performed in the physical pretreatment whereas white rot fungus (*Ganoderma boninense*) was used in the biophysical pretreatment. Cellulase-producing fungi, *Aspergillus niger* and *Trichoderma reesei*, were exploited in this study. Although lignin content was reduced by two-fold after the biophysical pretreatment, the maximum production of cellulase occurred when only the physical pretreatment was employed on the substrate. It may be because the higher apparent crystallinity of cellulose in physical pretreatment triggers more cellulase production compared to that in biophysical pretreatment. The maximum cellulase activity was found to be 1.035 IU mL⁻¹ when water hyacinth was only physically pretreated.

Key words:

Aspergillus niger; cellulase, cellulose, pretreatment, *Trichoderma reesei*, water hyacinth

Introduction

Water hyacinth (*Eichhornia crassipes*) is known as a weed aquatic plant because it severely disrupts aquatic ecosystems and emerges due to eutrophication. The overgrowth of water hyacinth results in various ecological and socio-economic issues.¹ It has become a particular concern in Africa, Asia, and North America.² The International Union for Conservation of Nature (IUCN) has classified water hyacinth as one of a hundred invasive plant species.³ Presently, the plant is eliminated by burning, which serves as a short-term solution; the burning may even initiate another environmental problem. Water hyacinth is a lignocellulosic material, which can be converted into useful products.

Recently, conversion of agro-waste into practical fuel like bioethanol has received substantial interest because agro-waste is available in abundance. The techniques used in the conversion vary depend-

ing upon the type of biomass and the production costs. One of the most essential and expensive steps is the pretreatment process. The purpose of pretreatment is to disrupt the physical and chemical structure of biomass in order to make cellulose become more susceptible to further hydrolysis.⁴ Research of pretreatment methods for lignocellulosic materials is often performed either in laboratory or pilot scale.

Chemical and physical pretreatment methods for lignocellulosic biomass are widely used in several studies. Chemical pretreatment often involves large amounts of chemicals at high concentration in order to degrade lignin and allow cellulase-producing microbes to easily hydrolyze cellulose.^{5,6} Although chemical and physical methods do not require a long time, they consume large amounts of energy and generate chemical waste.⁷ Hence, this study is focused on a more environmentally friendly approach through a biological method to minimize the waste.

The use of microorganisms in both biological pretreatment and cellulase production is crucial.

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White rot fungus (*Ganoderma boninense*), a pathogenic fungi that affects oil palm tree, is often used in biological pretreatments.⁸ The *Ganoderma* family also possesses the ability to degrade lignin.⁹

Enzymatic hydrolysis is considered the most efficient and least polluting method to generate glucose from lignocellulosics. However, the expense of hydrolyzing enzyme, such as cellulase, is still costly. In addition, the production economics of bioethanol is largely dependent on the price of cellulase.¹⁰ Thus, further research should investigate the production of cellulase in order to reduce the cost.

Cellulase is the third largest industrial enzyme in the world. It has gained increasing interest, particularly in the bioconversion of lignocellulosic biomass.¹¹ Cellulase is heavily used in several industries including food, textile, paper, bioethanol, ink, and pulp processing.^{12,13} Only certain microorganisms can utilize cellulose as an energy source to produce a complete cellulase complex. The complete cellulase complex consists of three main components: endo- β -glucanase, exo- β -glucanase, and β -glucosidase. Commercial cellulase-producing microbes are generally limited to *Trichoderma reesei*, *Aspergillus niger*, *Thermomonospora fusca*, and *Bacillus* sp.¹⁴ Thus, the production of cellulolytic enzymes by *A. niger* and *T. reesei* from physically and biophysically pretreated water hyacinth are investigated in this study. The pretreatment effect is also examined by determining the alteration of structure composition and cellulose crystallinity after pretreatment. Moisture content and incubation period are the parameters that are observed and reported.

Materials and methods

Materials

All chemicals used in this study were obtained from Merck, Tbk, Jakarta-Indonesia in analytical grade. Potato dextrose agar (PDA) from Oxoid™, 3,5-dinitrosalicylic acid and CM-cellulose were purchased from Sigma-Aldrich (Singapore).

Preparation of microorganisms

White rot fungus (*G. boninense*) was isolated from uprooted oil palm given by the Department of Biology, University of North Sumatera. *A. niger* and *T. reesei* were obtained from Bandung Institute of Technology. The strains were cultivated on potato dextrose agar (PDA) slants/plates at 30 °C for 72 hours.¹⁵ A sterilized wire loop was used to collect *A. niger* or *T. reesei*. The loop was dipped in a liquid medium containing sucrose 22 % (w/v), (NH₄)₂SO₄ 1 % (w/v), KH₂PO₄ 1 % (w/v), and incubated at 30 °C.¹⁶

Preparation and pretreatment of water hyacinth

Water hyacinth was collected from the local water bodies at Lhokseumawe-Medan, Indonesia. The collected plant was subjected to physical and biophysical pretreatment as shown in the scheme (Fig. 1). The physical pretreatment was conducted through the combination of chopping, drying at 105 °C, and grinding to the size of 0.1–1 mm. The ground water hyacinth was stored in a dry container

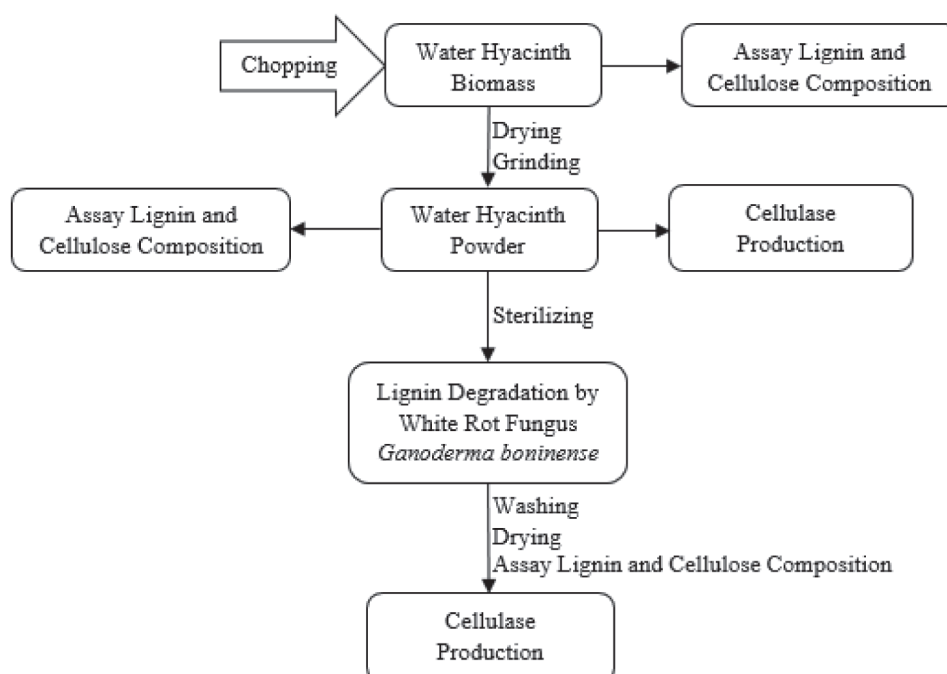


Fig. 1 – Pretreatment scheme

at room temperature. The composition of lignin and cellulose was examined prior to and after the physical pretreatment.

The biophysical pretreatment was conducted by using white rot fungus, *G. boninense*, to degrade lignin in water hyacinth. The 7-day biological degradation process was carried out with additional nutrient according to Mandel Weber medium¹⁷ at pH 5 and 70 % (w/v) moisture content. The sample was washed and dried after the process. The dried sample was assayed to determine lignin and cellulose content.

Compositional analysis

The composition of lignin and cellulose in water hyacinth was evaluated using Chesson method.¹⁸ Wide angle X-Ray Diffraction (XRD) was used to measure crystallinity of the substrate. The percentage of apparent crystallinity in water hyacinth after physical and biophysical pretreatments was measured by XRD Peak Height Method.^{19,20} The XRD measurements were performed using Philips Analytical System with PC-APD and Diffraction software (Bandung Institute of Technology).

Enzyme production

The pretreated water hyacinth was fermented using solid-state fermentation (SSF) method. The enzyme production experiments were carried out in 250-mL Erlenmeyer flasks containing 10 g of substrate. The effect of moisture content 65, 70, 75, 80, and 85 (% of wet mass) was studied to find the maximum cellulase production with Mandel Weber medium. The initial pH for medium was adjusted to 5.²¹ Then, the medium was autoclaved at 120 °C for 15 minutes, cooled, and inoculated with 15 % (w/w) suspension.²² The inoculated flasks were incubated in a temperature-controlled shaker at 30 °C^{23,24} for 3, 5, 7, 8, and 9 days. The effect of mono and mixed microbial cultures was studied to maximize cellulose production, and the appropriate microbe was applied to other variables. All experiments were prepared in duplicate.

Enzyme activity assay

The enzyme was extracted by adding 5:1(w/w) distilled water to the fermented substrate. The solution was centrifuged (Dynac II centrifuge, Clay Adams range 0–4000 rpm) at 2500 rpm for 15 minutes. The clear supernatant (crude enzyme) was subjected to measurement of cellulase activity according to modified International Union of Pure and Applied Chemistry (IUPAC) guidelines using 3,5-dinitrosalicylic acid (DNS) method.²⁵ Crude enzyme (0.5 mL) was added into 1.8 mL of substrate

solution (1 % CM-cellulose in 1.8 mL of 0.05 M phosphate citrate buffer, pH 5). Hydrolysis was performed by incubation at 30 °C for exactly 30 minutes. Glucose concentration was determined by DNS. The absorbance was measured using UV-1800 spectrophotometer (Shimadzu) with the maximum wavelength 503.3 nm. The amount of glucose released was determined by comparing it to the glucose standard curve. One IU mL⁻¹ was defined as the amount of glucose (μmol) per ml of enzyme solution per minute.

Results and discussion

Effect of physical and biophysical pretreatments on lignin degradation

The benefits of pretreatment for lignocellulosic biomass have been recognized for decades.²⁶ Although the pretreatment methods to produce cellulase have been studied extensively, only a few utilize water hyacinth as a substrate.^{24,27} In this study, two methods of pretreatment were employed to water hyacinth. Physical pretreatment was conducted by reducing the particle size by chopping and grinding. Meanwhile, the biophysical pretreatment was the combination of physical and biological pretreatments. The reduction of particle size breaks down cellulose crystallinity and increases surface area, which may allow hydrolysis to occur more rapidly.²⁸ The biophysical pretreatment was performed by exploiting white rot fungus to degrade lignin in the powdered biomass (from physical pretreatment). The biological process in biophysical pretreatment can remove lignin and hemicellulose, reduce crystallinity of cellulose, and increase porosity of the lignocellulosic materials.²⁹ In the present study, lignin content in water hyacinth was reduced by fermentation using white rot fungus, *G. boninense*. Fig. 2 illustrates the growth of *G. boninense* as the degradation process continued up to 7 days.

Fig. 2 shows that *G. boninense* grew vigorously on the water hyacinth substrate. As *G. boninense* infected the plant, the digestion of the cell wall began.^{8,30} Another study revealed that the other species of *Ganoderma*, *G. colossum*, also possessed the ability to degrade lignin.³¹

Lignin and cellulose contents were measured and compared. Table 1 reports the effect of pretreatments on the lignin and cellulose. As illustrated in Table 1, the amount of lignin after the biophysical pretreatment relatively decreases compared to that after the physical pretreatment. It demonstrates that *G. boninense* is also capable of degrading lignin within a short time. The time it took for *G. boninense* to degrade lignin was 7 days.



Fig. 2 – Biophysical pretreatment using *G. boninense* to degrade lignin in water hyacinth after (a) 2 days of incubation, (b) 7 days of incubation, and (c) washed and dried



Fig. 3 – Physical appearances of fungi in PDA agar plates/slants (a) left: *Trichoderma reesei*; right: *Aspergillus niger* (b) *Ganoderma boninense*

Table 1 – Lignin and cellulose contents (in % dry matter) of water hyacinth before and after pretreatments. The result was determined using Chesson method.

Water hyacinth biomass	Lignin (%)	Cellulose (%)
before pretreatment	5.99 ± 0.017	27.78 ± 0.012
after physical pretreatment	4.63 ± 0.023	29.91 ± 0.011
after biophysical pretreatment	2.90 ± 0.013	32.20 ± 0.021

In this study, lignin in lignocellulosic biomass is digested by the fungi's extracellular enzyme, which considerably enhances the microbes' accessibility to cellulose and potentially their cellulase production. According to other studies,^{32,33} the carbon source is one of the factors that influences cellulase enzyme production; the easily accessed cellulose would induce even more production. Water hyacinth is chosen as the substrate in the present study because it contains lower lignin composition compared to other lignocellulosic biomass.^{34,35} Since lignin binds cellulose in lignocellulosic structure, and thus inhibits cellulase production, a careful study of lignin composition is crucial.³⁶ Nonetheless, the cellulose structure in water hyacinth requires further study because other factors beside lignin may influence cellulolytic enzyme production.^{33,37}

Effect of different fungi cultures on cellulase production

The difference in fungi growth between Ascomycetes (*A. niger* and *T. reesei*) and Basidiomycetes (*G. boninense*) could be easily observed (Fig. 3). The hyphae of *A. niger* and *T. reesei* were spread

evenly throughout the plate, while the hyphae of *G. boninense* were crowded together in one mass.

The study of microbial cellulolytic activity is essential in enzyme production. Two strains of cellulase producers, *A. niger* and *T. reesei*, were utilized. Both fungi cultures were fermented individually, as well as co-fermented to determine their cellulase fermenting capabilities. The results are shown in Fig. 4.

According to Fig. 4, cellulase activity is much higher when the substrate is only physically pretreated. The mono-culture fermentation of either *A. niger* or *T. reesei* shows higher cellulase activities than that of the mixed culture fermentation.

A. niger is found to be a more suitable candidate than *T. reesei* due to its production stability of cellulase, as indicated by error bars in Fig. 4. *A. niger* and other cellulolytic fungi have the ability to penetrate through cellulosic substrates by hyphal extensions. The rate of cellulase production is directly proportional to the growth of the fungi in the substrate.³⁸ Ammonium sulfate, the nitrogen source used in this study, is the most preferable nitrogen supply for *Aspergillus* genus.^{39,40} Moreover, the mixed culture fermentation process shows lower cellulolytic activity than the mono-culture fermentation. The result follows a similar trend in another study.³⁷ They also reported that *A. oryzae* possessed higher cellulase activity than *T. reesei*.³⁷

A. niger was chosen as the fermentative microbe for further experiments as it had higher and more stable cellulase activity, and was easier to grow in PDA slants/plates than *T. reesei*.

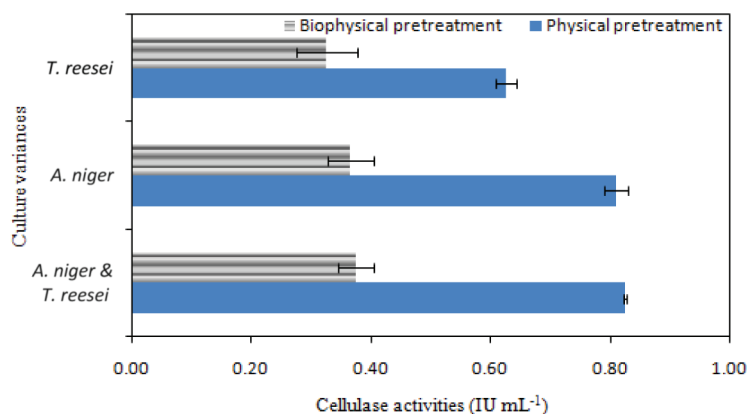


Fig. 4 – Cellulolytic activity of different fungi cultures obtained from the fermentation of pretreated water hyacinth at 30 °C after 7 days of incubation

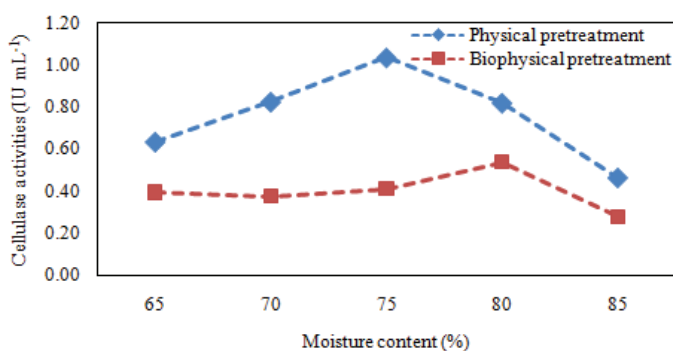


Fig. 5 – Effect of moisture content on cellulolytic activity at 30 °C after 7 days of incubation by *Aspergillus niger*

Effect of moisture content on cellulase production

Moisture content is an important factor for SSF since the microorganisms require a moist environment to ferment and sustain. Moisture content also has a significant effect on cellulase production.^{39,41} In this study, cellulase activity produced by *A. niger* was enhanced when the moisture content was higher than 70 %, as illustrated in Fig. 5. The optimized cellulase activities were 1.035 IU mL⁻¹ and 0.535 IU mL⁻¹ when the moisture contents were 75 % in physical pretreatment and 80 % in biophysical pretreatment, respectively. Both experiments were incubated for 7 days at initial pH 5.³⁵ The cellulase activity of the substrate from physical pretreatment is higher than that from the biophysical counterpart.

Moisture content and temperature are two of the most important factors for incubation because they are directly related to the heat problem in fungal growth.⁴² They could also play a role in the oxygen level of the substrate. In this study, the optimized incubation temperature (30 °C) according to Ilyas *et al.* (2011) was utilized even though the mesophilic fungi could grow well within the temperature range of 20–50 °C.⁴³

In addition to moisture content and temperature, pH can also influence the fungal growth and essentially enzyme production. The maximum cellulase production was observed in Juhasz *et al.* (2004) when the incubation pH was 5. The similar pH was also utilized as the optimized pH in other studies.^{15,44} However, the result did not show significant changes when the incubation pH was increased.²¹ Thus, the optimal incubation parameters in this study were 30 °C at pH 5.

The result from Fig. 5 indicates that cellulolytic activity, as a function of moisture content, has an optimum range between 65–80 % (w/w). The increase in cellulase activity at the specific moisture content from the experiment is relatively similar to other previous reports where lignocellulosic biomass was utilized as the carbon source, i.e. wheat bran, corn bran, kinnow peel, dry orange peel, sugar cane bagasse, and soybean hulls.^{15,37,45,46} Ilyas *et al.* (2011) were able to obtain 0.408 IU mL⁻¹ of cellulase enzyme activity at 70 % moisture content while using *V. mungo* plant as the sole carbon source.³⁹ However, the production of cellulase is higher when the water hyacinth (*E. crassipes*) is employed.

The decrease in the activity after the optimal range may be related to substrate swelling, nutrient diffusion, and solubility of solid substrates.⁴⁷ The excess of moisture content would lower the free air in the substrate, which would negatively affect oxygen transfer. Since oxygen is crucial to the microorganisms' growth and metabolism, the substrate should contain a suitable amount of water to maintain oxygen transfer.⁴¹

Effect of incubation period on cellulase production

The incubation period is one of the most critical aspects that affects enzyme production. At the optimized incubation period, the maximum activity was detected on the seventh day (Fig. 6). Then, the activity decreased on the eighth day and after.

The maximum enzyme activity was detected on the seventh day and began to decrease after the eighth day onward.^{44,48} The decrease in cellulase activity may be due to the cumulative effect of cellobiose, which is known to inhibit both endoglucanase and β -glucosidase, and deplete the nutrients.^{49–51}

The best results of enzyme production in this study occurred in the physical pretreatment substrate. Although the lignin content after the biophysical pretreatment had reduced, it did not indicate an increment in enzyme production as evident

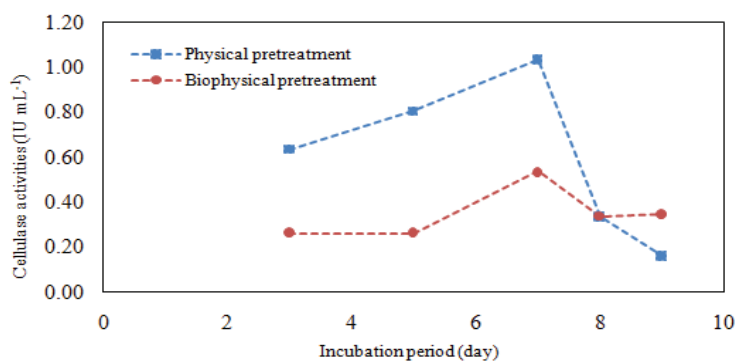


Fig. 6 – Effect of incubation period on cellulolytic activity by *Aspergillus niger*. The moisture contents for the physical and combined pretreatments were adjusted to 75 % and 80 %, respectively. The incubation temperature was 30 °C.

in Fig. 3 to Fig. 6. The crystalline structure of cellulose may positively influence the production of cellulase.

The maximum cellulase production from this study was 1.035 IU mL⁻¹ when the physically pretreated water hyacinth was incubated with *A. niger*. This result was proportionally similar to other cellulase production studies from other carbon source, i.e. ground nut shell. The higher cellulose composition (65.7 %) of ground nut shell caused the cellulase to be produced at 1.539 IU mL⁻¹ using *A. terreus*.⁴⁰ Jabasingh *et al.* (2014) used coir pith as carbon source, which contains similar cellulose composition to that of water hyacinth. The obtained cellulase enzymes production was 60.532 U mL⁻¹ using *A. nidulans*.³⁵ Although, several other conditions should be further investigated in cellulase production studies, water hyacinth remains the potential candidate for the carbon source in future research.

Crystallinity measurement of the biomass related to cellulase production

The results demonstrated that lower lignin content does not positively contribute to higher cellu-

lase production. One study suggests that cellulose crystallinity may significantly affect the production of cellulolytic enzyme.³⁷ Exposure of crystallinity would stimulate the production of cellulase from *Trichoderma reesei* and *Aspergillus oryzae*. This means that larger apparent crystallinity of cellulose would induce a higher production rate of exo- and endoglucanase from the microorganisms. In addition, the crystalline structure of the carbon source may also result in an increment of cellulolytic expression in many fungi.⁵²

The crystallinity of biomass can be determined using X-Ray Diffractometers (XRD). The XRD diffractograms in Fig. 7a and b measured the crystallinity of water hyacinth substrate after the physical and biophysical pretreatments.

The percentage of apparent crystallinity was calculated by comparing the intensity of the crystalline peak to the total intensity. The apparent crystallinity of water hyacinth after the physical and biophysical pretreatments was 56.9 %, and 53.6 %, respectively. The crystalline measurements demonstrated the organisms' abilities to break down crystalline content in water hyacinth and produce cellulase.

Conclusions

Water hyacinth could be utilized as the substrate for cellulase production. The best microbe to produce cellulase in this study was *Aspergillus niger* for both physical and biophysical pretreatments. Physical pretreatment gave higher cellulase production than biophysical pretreatment. The maximum cellulase activities after 7 days of incubation were 1.035 IU mL⁻¹ and 0.535 IU mL⁻¹ when the moisture contents were 75 % and 80 %, respectively, at 30 °C with initial pH 5.

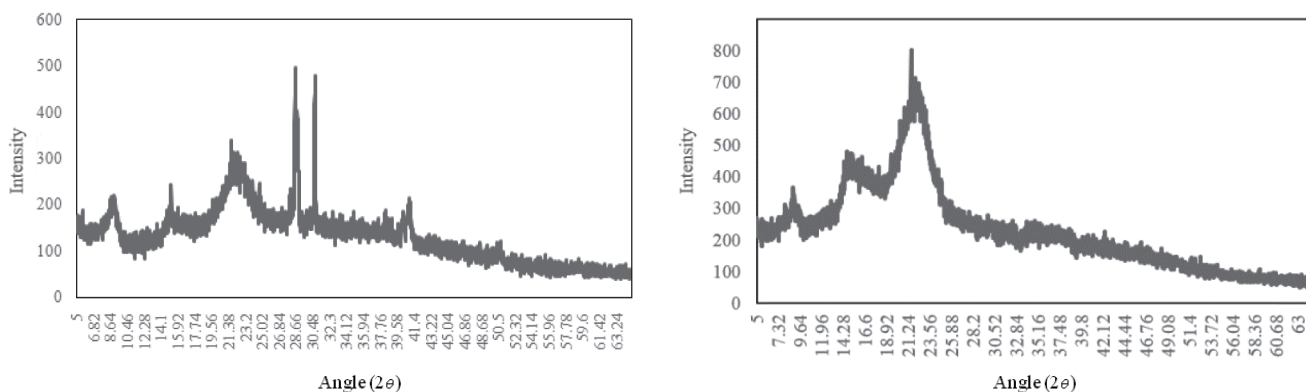


Fig. 7 – Diffractograms of water hyacinth after a) physical, and b) biophysical pretreatment

ACKNOWLEDGEMENT

The authors would like to acknowledge Bandung Institute of Technology, Indonesia Endowment Fund for Education, Jakarta, Indonesia, and Hungarian Scientific Research Fund (OTKA/NKFIH PD-108389) for financial support.

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