E-ISSN: 2655-8777

3Bio

JOURNAL OF BIOLOGICAL SCIENCE, TECHNOLOGY AND MANAGEMENT

Volume 2, No 1, 2020

School of Life Sciences and Technology
Institut Teknologi Bandung - Indonesia

3BIO: Journal of Biological Science, Technology and Management

Volume 2 • No 1 • 2020

This journal and individual articles are published at http://journals.itb.ac.id/index.php/3bio
a series imprint of the ITB Journal – an open access publisher.

ISSN 2655-8777 (Online)

3Bio: Journal of Biological Science, Technology and Management is a peer-refereed journal which is published twice a year. It is an interdisciplinary journal with its core in basic and applied life sciences and aims to address sustainability issues.

AIMS AND SCOPE

Journal of Biological Science, Technology and Management (3BIO) is the official journal of the School of Life Sciences and Technology, Institut Teknologi Bandung, Indonesia. 3BIO is an open access journal and published by ITB Journal. It is an interdisciplinary peer-reviewed journal in a wide aspect related to the field of life sciences and other related fields of study. The journal aims to promote scientific discourse and disseminate research on various branches and applications of bio-science, biotechnology and bio-based management.

This journal invites original empirical research, literature reviews, theoretical or methodological contributions, or short communications on, but not limited to, the following topics:

- Ecology and Biosystematics
- Microbiology
- Genetics and Molecular Biology
- Animal Development and Physiology
- Plant Development and Physiology
- Entomology
- Biomedical science
- Biochemistry
- Agronomy
- Forestry
- Bioengineering
- Bioethics
- Management of Biological Resources.

The journal also invites contributions from other associated disciplines.

ITB Journal (formerly Proceedings ITB), the official ITB scientific journal, has been published since 1961. It serves a vehicle for ITB's Faculty members and contributors from outside of ITB to publish research findings in science, technology and fine arts.

For more information about how to submit an article, please refer to our website: http://journals.itb.ac.id/index.php/3bio

COPYRIGHT

TABLE OF CONTENTS

3BIO: Journal of Biological Science, Technology and Management 2(1):1-6
e-ISSN: 2655-8777 https://journals.itb.ac.id/index.php/3bio

The effect of Paraquat Dichloride application on diversity and abundance of soil arthropods in the corn field

Tjandra Anggraeni[1]*, Taufikurahman[1], Meilisa[1], Yeyet Setiawati[1], Isma Fadilla[1], Asih Suryati[1]

[1)] School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*Corresponding Author: tjandra@sith.itb.ac.id
Received 29/05/2019
Accepted for publication 20/04/2020

Abstract

We investigated the effect of paraquat dichloride herbicide application on diversity and abundance of soil arthropods in a corn field. In addition to control (0 mL/L), four concentrations of paraquat dichloride were applied i.e. 3.33 mL / L, 4 mL / L, 4.66 mL / L and 5.33 mL / L. Sampling was carried out four times, namely at T_0 (before tillage), T_1 (after tillage), T_2 (after spraying herbicides), and T_3 (before harvesting) using a pitfall trap method. Soil arthropod samples were identified to the family level. The total number of individuals was then analyzed to obtain species richness, species dominance index, species diversity index and similarity index of Sorensen. The result showed that paraquat dichloride did not give any significant effect (p>0.05) on the number of individuals, species richness, species dominance index, diversity index and Sorensen similarity index of soil arthropods. The composition of soil arthropods in the control and in the treatment with the highest concentration shows a high level of similarity.

Keywords: soil arthropods, paraquat dichloride, diversity, abundance

1. Introduction

Herbicide is one type of pesticide that is used to eliminate or inhibit the growth of unwanted plants that are commonly known as weed. Herbicide changes the influence of chemicals in weed tissues, which can destroy the said tissues or damage the physiological system needed for life or growth. When processes of respiration, photosynthesis, cell division, and elongation of cells are disturbed, weeds will deplete energy reserves [1].

Paraquat dichloride is a bipyridylium herbicide group, which is also known by the name 1,1'-dimethyl-4,4'-bipyridinium dichloride or N, N'-dimethyl-4,4'-bipyridinium dichloride [2]. Paraquat dichloride has an empirical formula of $C_{12}H_{14}N_2Cl_2$, and is commonly used as an herbicide to control weed growth in agricultural and plantation areas in various regions in Indonesia. Paraquat dichloride is very soluble in water and is a contact herbicide that works in the leaves, particularly in the chloroplasts. Paraquat absorbed by plants will inhibit photosynthesis, more specifically the photosystem I, which causes the formation of reactive oxygen species (ROS) such as superoxide radicals (O^{2-}) or peroxide acid (H_2O_2), which will then attack cell membranes [2]. Oxidative lipid degradation (lipid peroxidation) is one of the parameters of damage caused by an increase in the number of ROS. This lipid degradation occurs as a result of the reaction process between unsaturated fatty acids in cell membranes and ROS to form lipid hydroperoxide (lipid hydroperoxides) [2]. The accumulation of lipid hydroperoxide in the membrane can disrupt the function of cell membranes, resulting in a loss of cell permeability and the occurrence of leakage [3].

A large amount of herbicide use in agriculture has been known to potentially cause agricultural land and water pollution [4, 5] and pose a threat to the health of animals [6, 7] and humans [8]. In non-target insects, Cousins et al. [9] reported the decreasing size of oenocytes of honeybee larvae after being exposed to very small concentrations of paraquat. Studies related to insect physiology also show that paraquat can decrease the number of the emergence of adult insects

https://doi.org/10.5614/3bio.2020.2.1.1

and compromise the ovipositioning ability of a parasitoid wasp, *Diaeretiella rapae* [10]. More than 95% of herbicide use is exposed to water, plants and non-target organisms such as soil arthropods [11]. Soil arthropods are particularly important in the process of breaking down organic matter, mineralizing plant nutrients, increasing soil aggregation, changing soil physical properties, stimulating succession, controlling pests and as indicators of pollution [12].

The impact of herbicide application on biodiversity and abundance of soil arthropods is pertinent, since it will compromise the sustainability of the ecosystem. A recent study related to that of ours was carried out in oil palm plantation [13], whereas in this study, the effect of the application of paraquat dichloride on the diversity and abundance of soil arthropods was carried out in a corn field.

2. Methodology

This research was conducted at the ITB Research Garden, Haurgombong Village, Sumedang as well as the Entomology Laboratory of SITH ITB from July to December 2018. Since the recommended concentration of paraquat dichloride was 4mL/L and a certain degree of error in applying this usually occurred among farmers, we experimented on a variety of paraquat dichloride concentrations, i.e. 0 mL/L (control), 3.33 mL/L, 4 mL/L, 4.66 mL/L, and 5.33 m/L. Each of the concentrations was applied on the sample plots within 35 days after planting. The size of the sampling plot was 5 m x 4.5 m and each treatment was applied in five plots. Sampling was carried out four times, namely at T_0 (initial conditions prior to tillage), T_1 (after tillage), T_2 (after pesticide spraying), and T_3 (before harvest).

A sampling of soil arthropods was made using a modified pitfall trap method [14], by installing a plastic cup containing detergent solution in the soil with the top opened and placing the cup in such a way that its top edge aligned with the surface of the soil. Each plot consists of three pitfall traps. Traps were set for 24 hours. Trapped arthropods were transferred into a plastic bag containing 70% of alcohol solution.

The identification of arthropods was carried out to the family level, using a stereo microscope and McGavin's arthropod identification book [15]. The data obtained were then analyzed by determining the Shanon-Wiener diversity index (H'), Simpson dominance index (D), and Sorensen (S) similarity index using the following formula:

$$\text{Shannon Index } (H') = -\Sigma\, p_i (\ln p_i),\ p_i = \frac{ni}{N} \quad (1)$$

$$\text{Simpson Index } (D) = \Sigma \frac{n_i (n_i-1)}{N (N-1)} \quad (2)$$

$$\text{Simpson Diversity Index} = 1-D \ \left(\textit{Simpson's reciprocal index} = \frac{1}{D}\right) \quad (3)$$

$$\text{Sorenson Index: } C = \frac{2j}{(a+b)} \quad (4)$$

Index:

p_i = proportion of individual species i
n_i = number of individual species i
N = total number of individuals
a = number of individuals at location A
b = number of individuals at location B
j = number of individuals at locations A and B

3. Results and discussion

3.1. Population dynamics of soil arthropods

Formicidae was the predominant group in the area for all sampling times, with the number of individuals reaching an order of hundreds (**Table 1**). Different result was reported in oil palm plantation with Formicidae being the dominant group only at week-0 and 4, whereas the Acarine was mostly found at week-2 and 8 [13]. Other families of soil arthropods showed a low number of individuals, most probably due to dry conditions. The event of rainfall at T_3 significantly increased the number of individuals of almost all families, especially Gryllidae, Araneidae, Pyrrhocoridae and Tenebrionidae. The application of paraquat dichloride did not give any significant effect on the number of individuals of the arthropods, as shown in **Table 1**.

3.1.1. Number of individuals

Total number of individuals of soil arthropods fluctuated from T_0 to T_3, without any specific trend. The highest number of individuals was found at T_2 sampling, which is after an herbicide application using 4.66 mL/L of paraquat dichloride. This shows that paraquat dichloride did not have a significant effect on the individual number of soil arthropods (see **Figure 1**).

3.1.2. Species richness

A comparison of the richness of soil arthropod species at T_0, T_1, T_2, and T_3 is listed in **Figure 2**. The richest species of soil arthropods are found at the time of T_3 (before harvest). Species richness shows the diversity of soil arthropods, which may be related to the health of the soil ecosystem. Species richness is among the factors that influence species diversity. After tillage (T_1), species richness of soil arthropods decreased in all treatment groups (**Figure 2**). This might occur since the soil was dried out without any standing crops left at the time of tillage, resulting in a disturbed habitat and decreasing soil arthropod richness [13,16].

https://doi.org/10.5614/3bio.2020.2.1.1

Table 1 Soil Arthropod Families Population Dynamics at T_0, T_1, T_2 and T_3

	Arthropod families	Number of individuals			
		T_0	T_1	T_2	T_3
1.	Acrididae	9	6	7	16
2.	Alydidae	2	0	0	0
3.	Araneidae	16	3	2	36
4.	Blatellidae	2	0	0	0
5.	Carabidae	2	0	0	4
6.	Cicadidae	10	1	2	7
7.	Chrysomelidae	1	0	0	5
8.	Clubionidae	0	0	4	14
9.	Forficuldae	0	0	0	4
10.	Formicidae	279	260	412	178
11.	Gryllidae	10	3	7	92
12.	Hesperiidae	0	3	2	0
13.	Ichneumonidae	0	0	3	0
14.	Libellulidae	0	0	0	2
15.	Muscidae	1	1	0	3
16.	Mycetophilidae	0	1	4	3
17.	Pentatomidae	1	0	0	9
18.	Pyrgomorphidae	0	0	3	0
19.	Pyrrhocoridae	0	1	1	36
20.	Rhinotermitidae	0	0	0	1
21.	Scarabaeidae	0	0	0	1
22.	Syrphidae	0	3	3	3
23.	Tenebrionidae	15	3	6	24
24.	Vespidae	2	1	1	1

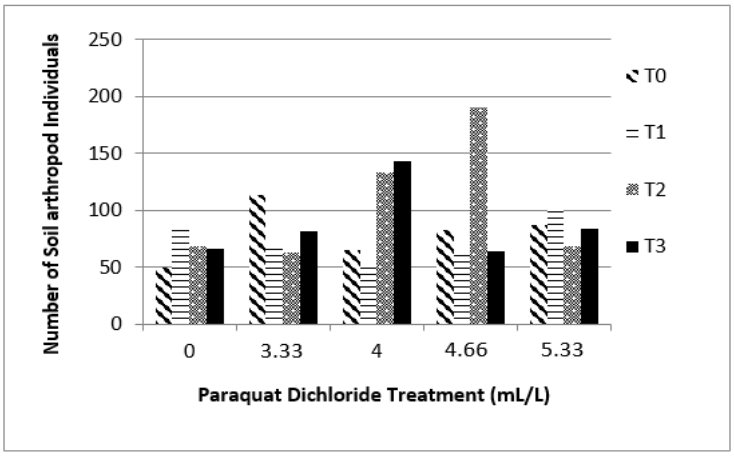


Figure 1 Comparison of the Number of Individual of Soil Arthropods at T_0, T_1, T_2 and T_3

https://doi.org/10.5614/3bio.2020.2.1.1

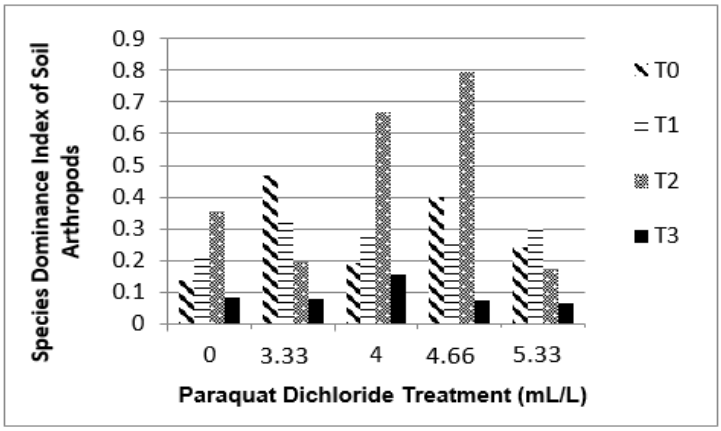


Figure 2 Comparison of abundance of soil arthropod species at T_0, T_1, T_2, and T_3

After spraying herbicide (T_2), the richness of soil arthropod species in the treatment group did not show any specific trend, which indicated that paraquat dichloride did not have a significant effect on the species richness of soil arthropods. Species richness of soil arthropods increased towards harvest (T_3) to a point higher than T_0. During the T_3 data collection, the rain had begun to fall. Air and soil temperatures decreased and were lower than the temperatures at T_0, T_1, and T_2. Soil moisture in the treatment plot also increased on T_3. Corn plants began to flourish, along with the surrounding weeds. This condition supports the survival of soil arthropods in the corn field, hence an increase in the species richness of soil arthropods. However, based on our One-Way ANOVA analysis, the herbicide paraquat dichloride did not have a significant effect on the richness of soil arthropod species ($p = 0.958$). Accordingly, Tukey's post-hoc test also showed that the species richness of soil arthropods between treatments did not differ significantly.

3.1.3. Species dominance index

A comparison of the dominance index of soil arthropod species at T_0, T_1, T_2, and T_3 is shown in **Figure 3**. The Simpson dominance index measures the presence of dominant species in an ecosystem. Higher index value indicates that the abundance of each species in the ecosystem is not evenly distributed. The low evenness of these species results in a low species diversity in the area. The dominance index of arthropods reached the highest value in treatment D (4.66 mL / L) at T_2 (an index value of 0.794), which showed a high degree of dominance (**Figure 3**). With T_1 and T_2, the dominance index values for the treatments do not correspond to any certain trend. However, at T_3 (before harvest) the dominance index value in all treatment groups decreased. It can therefore be concluded that at the time of T_3, the diversity of soil arthropods increased. However, based on the One-Way ANOVA analysis, paraquat dichloride did not have a significant effect on the dominance index of soil arthropod species ($p = 0.631$).

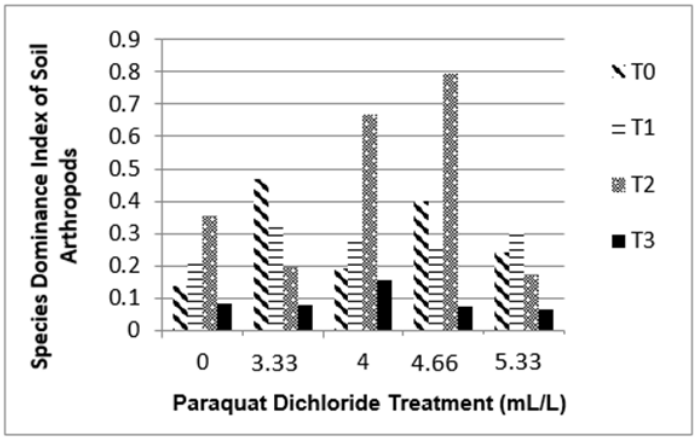


Figure 3 Comparison of the dominance index of soil arthropod species on T_0, T_1, T_2, and T_3

https://doi.org/10.5614/3bio.2020.2.1.1

According to the Tukey post hoc test, the dominance index of soil arthropod species between treatments did not differ significantly.

3.1.4. Species diversity index

Comparison of the diversity index of soil arthropod species at T_0, T_1, T_2, and T_3 is shown in **Figure 4.** Species diversity index shows the diversity of species in a certain habitat. Diversity is influenced by both species richness and evenness, the latter can be measured through the dominance index of species. One one hand, at T_1 and T_2, the diversity index value in the treatment does not have a certain trend. Spraying herbicide may not have a significant effect on the species diversity index at the time of T_2. On the other hand, at T_3 (before harvest), the diversity index value in all treatment groups experienced an increase (**Figure 4**).

Based on the One-Way ANOVA analysis conducted, the use of paraquat dichloride did not result on any significant effect on the diversity index of soil arthropod species ($p = 0.818$). Similarly, Tukey's post-hoc test also shows no significant difference between the diversity index values of soil arthropod species.

3.2. Sorensen similarity index

The Sorensen similarity index value between control (A) and the highest concentration treatment groups (E) is shown in **Figure 5**. The Sorensen (IS) similarity index between the control group and the highest concentration of paraquat dichloride herbicide treatment at T_0, T_1, T_2, and T_3 (with a value exceeding 50%) showed that the composition of soil arthropods in the two treatment plots were highly similar (**Figure 5**). Based on the results of identification (**Table 1**), Formicidae is the most common family of arthropods at T_0, T_1, T_2, and T_3, compared to other families.

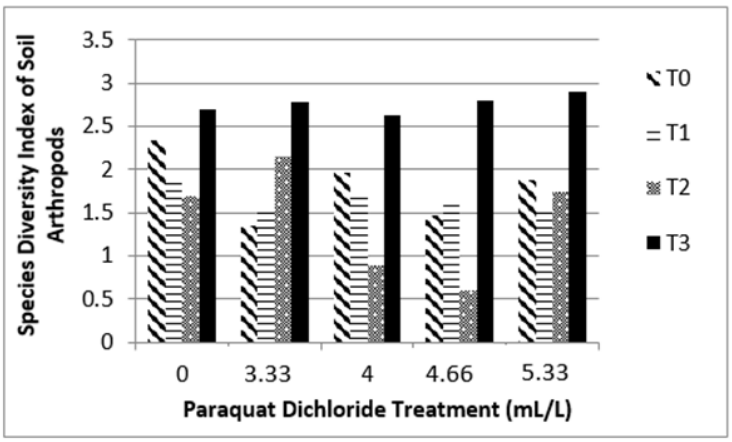


Figure 4 Comparison of diversity index of soil arthropod species at T_0, T_1, T_2 and T_3

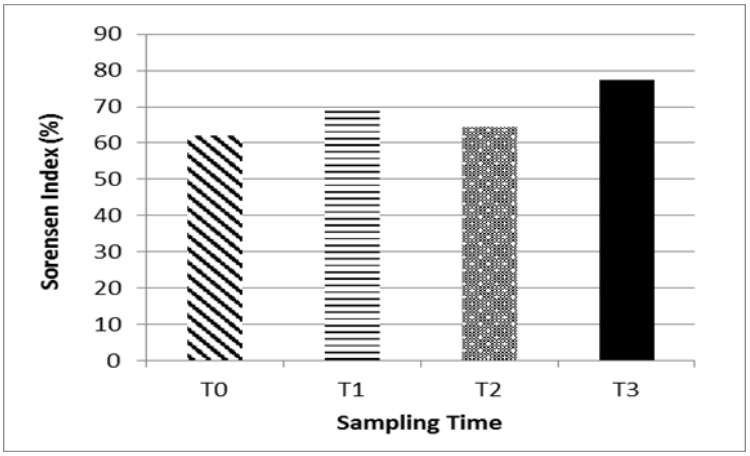


Figure 5 Sorensen similarity index value between control and treatment groups with the highest paraquat dichloride concentration at T_0, T_1, T_2 and T_3

https://doi.org/10.5614/3bio.2020.2.1.1

4. Conclusion

From the results of the study, it can be concluded that the herbicide paraquat dichloride has no significant effect ($p > 0.05$) on individual numbers, species richness, species dominance, and diversity indices of soil arthropod species, whereas the Sorensen similarity index shows that the composition of soil arthropods in the control group and the treatment group with the highest concentration shows a high similarity, as indicated by an index value of more than 50%.

Acknowledgments

This research is funded by the Directorate of Fertilizers and Pesticides, the Directorate General of Agricultural Infrastructure and Facilities, Ministry of Agriculture of the Republic of Indonesia, to which we express our gratitude. We also thank the executive director of ITB Campus Jatinangor to allow the use of the Haurgombong Research Garden land to carry out the implementation of this research, and the Dean of SITH ITB who granted a permission and administrative assistance to conduct this research.

References

[1] Riadi, M. 2011. Mata Kuliah : Herbisida Dan Aplikasinya. Jurusan Budidaya Pertanian. Fakultas Pertanian Universitas Hasanuddin.

[2] JinHua Z, Keli Y, ZhongGui Z, WuSheng J, DongHua L. Antioxidant response system and chlorophyll fluorescence in chromium (VI)-treated Zea mays L. seedlings. Acta Biologica Cracoviensia. Series Botanica. 2009; 51(1): 23-33.

[3] Saelim S, Zwiazek J.J. Preservation of thermal stability of cell membranes and gas exchange in high temperature acclimated Xylia xylocarpa seedlings. Journal of Plant Physiology. 2000; 156(3): 380-385. DOI: 10.1016/S0176-1617(00)80077-2

[4] Ikpesu T.O. Assessment of occurrence and concentrations of paraquat dichloride in water, sediments and fish from Warri River Basin, Niger Delta, Nigeria. Environmental Science and Pollution Research. 2015; 22(11): 8517–8525. DOI: 10.1007/s11356-014-3993-2

[5] Martins D.A, Simões M, Melo L. Adsorption of paraquat dichloride to kaolin particles and to mixtures of kaolin and hematite particles in aqueous suspensions. Journal of Water Security. 2015; 1: 25-36. DOI: 10.15544/jws.2015.003

[6] Lalruatfela P.L, Samanathan M, Ingole R.S, Dhama K, Joshi M.V. Toxicopathology of paraquat herbicide in female wistar rats. Asian Journal of Animal and Veterinary Advances. 2014; 9(9): 523-542. DOI: 10.3923/ajava.2014.523.542

[7] Muslim C, Santi Nurul Kamila, S.N. The effect of paraquat inhalation on parkinsonism, organ morphology and anatomy of mice and its recovery with the application of etliringea hemisphaerica (blume, r.m. smith) crude extract. IOP Conference Series: Earth and Environmental Science. 2018; 130: 012036. DOI :10.1088/1755-1315/130/1/012036

[8] Sondhia S. Herbicides residues in soil, water, plants and non-targeted organisms and human health implications: an Indian perspective. Indian Journal of Weed Science. 2014; 46(1): 66–85.

[9] Cousin M, Silva-Zacarin E, Kretzschmar A, El Maataoui M, Brunet J-L, Belzunces L.P. Size changes in honey bee larvae oenocytes induced by exposure to paraquat at very low concentrations. Plos One. 2013; 8(5): e65693. DOI: 10.1371/journal.pone.0065693

[10] Xu R, Mortimer P.E, Kuang R.P, He J, Zhang W.D, Yin, F. Sublethal impact of paraquat on the life span and parasitic behavior of Diaeretiella rapae M'Intosh. Journal of Environmental Science and Health B. 2013; 48(8): 651–657. DOI: 10.1080/03601234.2013.778597

[11] Ardiwinata A, Nursyamsi D. Residu pestisida di sentra produksi padi di Jawa Tengah. Jurnal Pangan. 2012; 21(1), 39-58.

[12] de Barros E.C, Ventura H.V, Gontijo P.C, Pereira R.R, Picanço M.C. Ecotoxicological study of insecticide effects on arthropods in common bean. Journal of Insect Science. 2015; 15(1): 1-14. DOI: 10.1093/jisesa/ieu172

[13] Dadang, Hartono A, Nurulalia L, Poernomo B, Soekarno W. Effects of paraquat dichloride application on soil arthropods and soil chemical and physical properties in oil palm cultivation. Journal of International Society for Southeast Asian Agricultural Sciences (ISSAAS). 2019; 25(2): 174-184.

[14] Ward D.F, New T.R, Yen A.L. Effects of pitfall trap spacing on the abundance, richness and composition of invertebrate catches. Journal of Insects Conservations. 2001; 5: 47-53.

[15] McGavin G. C. Smithsonian handbooks of Insects—spiders and other terrestrial arthropods. Dorling Kindersley; 2002. 256 p.

[16] Suhardjono Y.R, Deharveng L, Bedos A. Biologi, Ekologi, Klasifikasi Collembola (ekor pegas). Vegamedia Bogor; 2012.

https://doi.org/10.5614/3bio.2020.2.1.1

e-ISSN: 2655-8777 https://journals.itb.ac.id/index.php/3bio

The Physiological Responses of Water Hyacinth (*Eichhornia crassipes (*Mart). Solms) and Water Lettuce (*Pistia stratiotes* L.) as Trivalent Chromium Bioaccumulator

Rizka Purnamawati [1]*, Taufikurahman[1], Andira Rahmawati[1], Chalvin Rura Putra[1], Din Dzakamala F.Rahmatilah[1], Findy Ashgi[1]

[1)] School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*Corresponding Author: taufik@sith.itb.ac.id
Received 31/12/2019
Accepted for publication 21/4/2020

Abstract

Chromium is one of the heavy metals used in industrial fields, i.e., metallurgical industry, chemical industry, heat-retardant, and leather tanning industry. Untreated wastewater from these industries can pollute rivers and threaten the aquatic ecosystem. Some aquatic plants such as water hyacinth (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes*) have been known as metal hyperaccumulators and can be used as phytoremediator for polluted water. This aim of the study is to determine the physiological and morphological responses of water hyacinth and water lettuce plants treated by different concentrations of trivalent chromium and to study which plant is effective for trivalent chromium removal. The experiment was conducted for 2 week at screen house. After two weeks, the plants were harvested and the weight was measured. The samples were separated into shoot and root and were analyzed for Cr content, chlorophyll, proline and CAT enzyme activity. The results of this study indicated that the levels of water hyacinth chromium in roots and leaves are lower (863.988 mg/Kg, 899.126 mg/Kg, 685.877 mg/Kg for treatment 40 ppm, 80 ppm, and 120 ppm respectively) than the levels of chromium in water lettuce (1584.264 mg/Kg, 1660 mg/Kg, 1413 mg/Kg for treatment 40 ppm, 80 ppm, and 120 ppm respectively). Physiological parameter, i.e., chlorophyll and proline levels in water hyacinth and water lettuce from all concentration treatment did not differ significantly (P> 0.05). The activity of the catalase enzyme in water hyacinth and water lettuce decreased with increasing chromium levels. The highest catalase enzyme activity was observed in control treatment of water lettuce (1.61 unit/mg) and 40 ppm treatment of water hyacinth (1.006 unit/mg). The highest biomass dry weight of both plants was found in plants with control treatment (15.38 gr and 8.48 gr for water hyacinth and water lettuce respectively). Therefore, we concluded that water lettuce is better for trivalent chromium removal than water hyacinth.

Keywords: bioaccumulator plants, heavy metal, water hyacinth, water lettuce

1. Introduction

Chromium (Cr) is one of the environmental pollutants originated from various industrial wastes. For instance, chromium (VI) and chromium (III) are used in the plating, textile, leather tanning, and wood preservation industries [1]. In general, chromium has two main forms in the soil environment [2-3], i.e., the dominant form chromium (III) that is under a reduced conditions, immovable, and less toxic to plants, and the oxidized form chromium (VI) that is very easy to move and carcinogenic to humans [3]. Changes in the concentration of Cr (VI) on soil can affect its absorption by plants, caused inhibited growth, disruption of photosynthesis and respiration, absorption of

https://doi.org/10.5614/3bio.2020.2.1.2

minerals, enzyme activity, and ended with damage to membrane lipids and plant DNA [4].

Water hyacinth including the Pontederiaceae family is found in the tropics and subtropics. Water hyacinths are classified as aquatic weeds that can adapt to changes in the environment and multiply rapidly. The ideal growing place for water hyacinth plants is shallow and turbid waters, with temperatures ranging from 28-30 °C and pH conditions ranging from 4-12. In deep and clear waters in the highlands, these plants are difficult to grow. Water hyacinth can suck water and vaporize it into air water through the transpiration process [5].

Pistia stratiotes are sometimes used as decorative plants in a pond and play an important role for phytoremediation of heavy metals, chemical products and radioactive material in water [6]. Different accumulations of Cr in parts of the plant have been reported. The root is the main organ in plants that is directly related to the absorption of nutrients from the environment, including heavy metal like Cr, Pb, Ni, etc. In beans, at least 0.1% of the total Cr that accumulates was found in the seeds, whereas the concentration in the roots reached 98% of the total absorption of Cr [7]. The amount of Cr accumulations (160-350 mg Cr / kg of dry weight at the root and reaching 1.6-2.0 mg Cr / kg dry weight on shoots) was observed for cauliflower, green vegetables, and chili. A high capacity to accumulate Cr was shown by water hyacinth (*Eichhornia crassipes*) with up to 6 mg/ day dry weight in the roots of this plant that grows at 10 ppm Cr [VI].

A study reported the toxicity of Cr on plant growth and development (i.e., change in germination process followed by changes in root, stem and leaf growth) can affect the total production of plant dry weight [8]. The inhibition of chlorophyll biosynthesis has also been reported in land plants [8-9]. For example, barley seedlings grown at 100 mM Cr showed a 40% lower growth rate [8]. Chromium also damaged the plants' physiological processes, i.e., photosynthesis, absorption of water, and nutrients absorption. Metabolic changes also occur with the production of enzymes and metabolites or by producing Reactive Oxygen Species (ROS) [10].

Heavy metals can have an ecological impact on water bodies leading to increased nutrient load especially if they are essential metals. These metals in the effluent may increase the fertility of water leading to eutrophication which in open water can progressively lead to oxygen deficiency, algae blooms, and death of aquatic life [11].

Chlorophyll and proline are some examples of plant metabolism antioxidant products [12] which play a role in the major metal detoxification mechanisms in plants [13]. Environmental changes such as pollution from industrial waste will alternate the physiological responses of the plant. The implication of this study is to study the changes in physiological plants as a trivalent chromium bioaccumulator agent.

2. Materials and methods

2.1. Plant material and experimental design

Water hyacinth and water lettuce with almost the same size for each plant (based on weight) was collected from a pond in ITB campus and with an analytical grade of Cr3+ (CrCl3). The experimental design consists of four treatments, i.e., control, Cr3+ 40 ppm, 80 ppm, and 120 ppm.

There were three replicates for each treatment. Twelve plants of each were placed in 10 L of medium. The experiment was conducted for 2 weeks at the screen house. After two weeks, the plants were harvested and weight was measured. The samples were separated into shoot and root and were analyzed for Cr content, chlorophyll, proline and CAT enzyme activity.

2.2. Cr content in the media, roots, and leaves

Cr content in the leaves and roots was measured by concentrating the sample with nitric acid (HNO3) [14]. The accumulation of Cr in *Eichhornia crassipes* and *Pistia stratiotes* was determined by calculating the concentration of chromium in its water, roots, and leaves. A comparison between the root/leaves concentration and concentration in water is known as Bio-Concentration Factor (BCF). BCF in leaves and roots was calculated to determine how much metal concentration in leaves and roots was assumed to be from the environment [15]. The formula used to calculate BCF was as follows:

$$\text{BCF} = \frac{\text{Cr in roots or leaves (mg/Kg)}}{\text{Cr in media (mg/Kg)}} \quad (1)$$

A comparison between the metal concentrations in leaves and roots known as Translocation Factors (TF) was also calculated. TF values were calculated to determine the displacement of metal accumulation from root to shoot [16]. TF values were calculated using the formula:

$$\text{TF} = \frac{\text{Cr in leaves (mg/Kg)}}{\text{Cr in roots (mg/Kg)}} \quad (2)$$

The differences between BCF and TF values were then used to calculate Phytoremediation (FTD) [16]. FTD was calculated using the formula:

$$\text{FTD} = \text{BCF} - \text{TF} \quad (3)$$

2.3. Chlorophyll content

Measurement of chlorophyll levels was carried out based on [17]. Fresh leaf samples were weighed 0.1 grams and then

https://doi.org/10.5614/3bio.2020.2.1.2

crushed using 80% acetone. The chlorophyll solution was then filtered into chlorophyll filtrate and then taken for absorbance measurements using a spectrophotometer at a wavelength of 645 nm and 663 nm. Chlorophyll levels were calculated using the formula:

$$\text{Total chlorophyll (mg/g)} = \frac{8{,}02\ A663 + 20{,}2\ A645}{1000\ FW} \text{ x V} \qquad (4)$$

V = sample volume
A = absorbance value
FW = sample fresh weight (g)

2.4. Proline levels

Proline level measurement was carried out by measuring the absorbance using a spectrophotometer at a wavelength of 520 nm [18]. Proline levels were calculated based on the proline standard curve based on fresh weight using the formula:

$$\mu\text{mol Prolin/g FW} = \frac{\mu\text{g Prolin x mL toluene}}{115.5} x \frac{5}{\text{g sample}} \qquad (5)$$

115.5 = proline molecule weight

2.5. Catalase enzyme activity (CAT)

CAT enzyme activity was measured based on the method carried out by [19]. CAT enzyme activity was measured using a spectrophotometer by measuring changes in sample absorbance at a wavelength of 240 nm as a consequence of H2O2 degradation. Readings of absorbance numbers were done every 30 seconds for three minutes. The activity of catalase enzyme was calculated using the formula:

$$\text{Volume activity (unit/mL)} = \frac{\Delta A\ x\ Vq}{0{,}0436\ x\ Vs} \qquad (6)$$

$$\text{CAT enzyme activity (unit/mg)} = \frac{\text{volume activity}}{\text{fresh weight}} \qquad (7)$$

ΔA = Absorbance at third minute - Absorbance at initial minute
Vq = Volume reaction in kuvet (ml)
Vq = Volume sample (ml)

2.6. Statistical analysis

Data were statistically analyzed using a statistic software IBM SPSS Statistics version 25. One-way ANOVA was used for proline, catalase enzyme, and chlorophyll content analysis. The results were presented in graphs. Data from different treatments and control were compared by Duncan's multiple range test (DMRT) at $p < 0.05$.

3. Results

3.1. Cr Level on Roots, Leaves and Medium

According to the result (**Figure 1** and **Figure 2**), the Cr content in the roots of water lettuce and water hyacinth has increased from control to the concentration of 80 ppm, but at the concentration of 120 ppm, it has decreased.

Figure 3 showed the ability of water hyacinth and water lettuce as a phytoremediation agent. The highest FTD value obtained from water lettuce root at Cr level 40 ppm, 80 ppm, and 120 ppm.

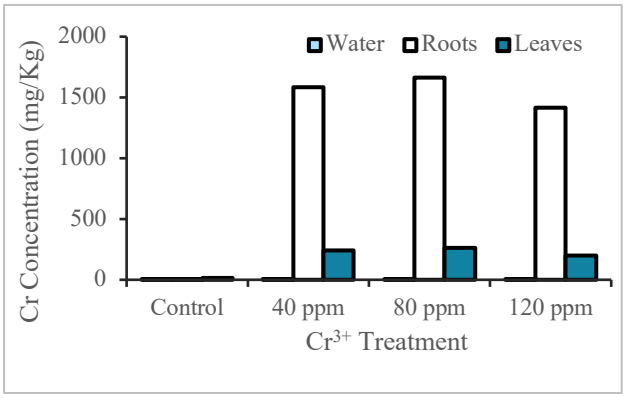


Figure 1 Analysis of Cr in water lettuce

3.2. Chlorophyll Level

Figure 4 showed that at concentrations of 0 to 80 ppm, the chlorophyll content in water hyacinth is above the chlorophyll content of water lettuce, while at the concentration of 120 ppm, the chlorophyll content of water lettuce is slightly higher. The statistical test showed no significant difference between the chlorophyll content of the two plants for each different Cr concentration ($P>0.05$).

3.3. Catalase Enzyme Activity

Both water lettuce and water hyacinth plants showed decrease in CAT enzyme activity along with the increase in Cr metal in water media (**Figure 6**).

https://doi.org/10.5614/3bio.2020.2.1.2

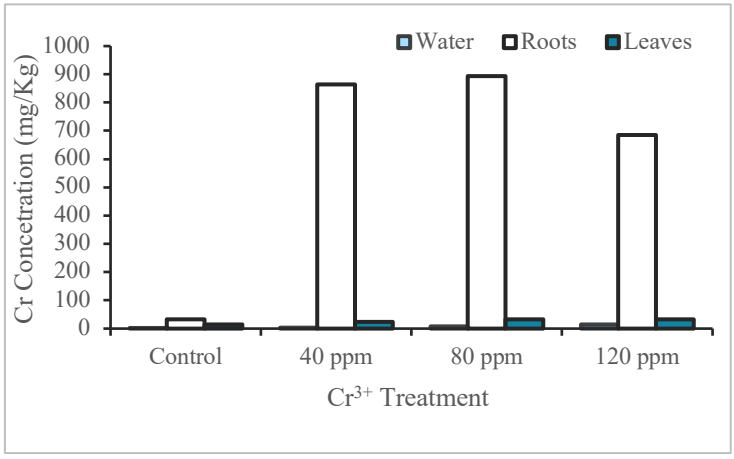


Figure 2 Analysis of Cr in water hyacinth

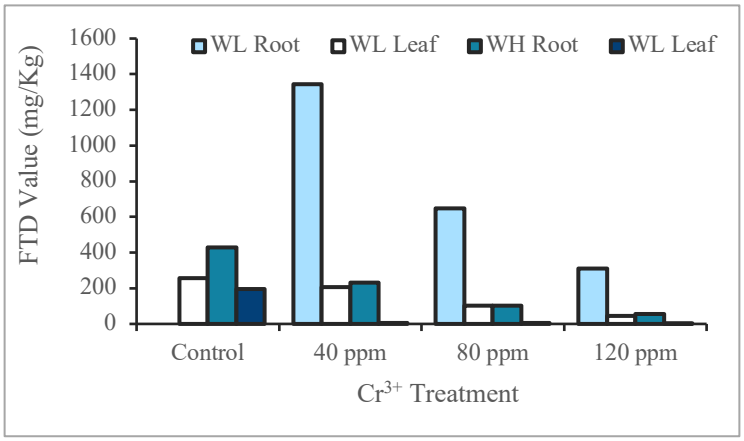


Figure 3 Graph of FTD value in water hyacinth and water lettuce

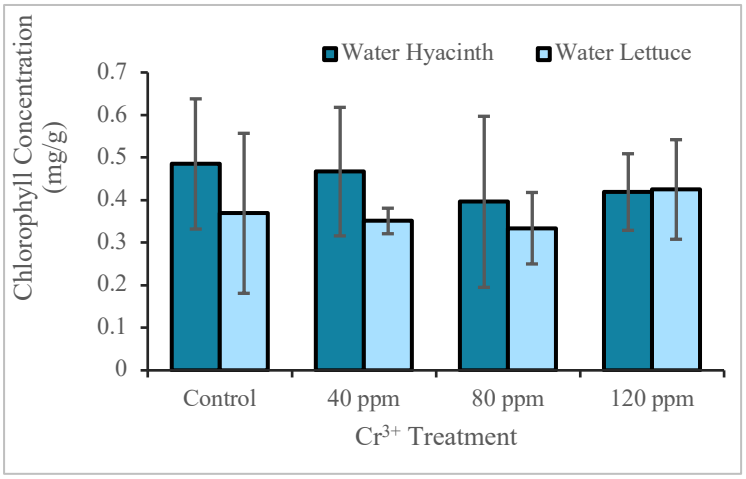


Figure 4. Graph of chlorophyll level in water hyacinth and water lettuce

https://doi.org/10.5614/3bio.2020.2.1.2

Figure 5 Comparison of water hyacinth in control treatment and 80 ppm treatment

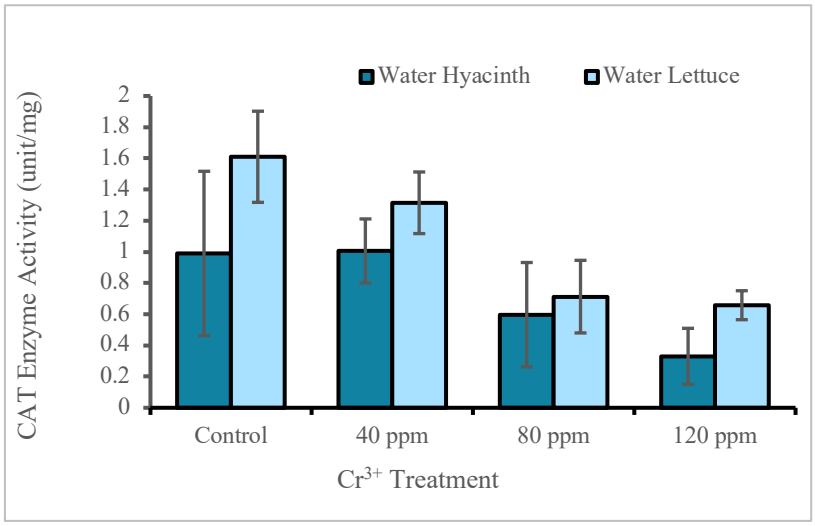


Figure 6. Graph of CAT enzyme activity in water hyacinth and water lettuce

3.4. Plant Biomass

In water hyacinth plants, the sample with 0 ppm treatment (control), has the biggest difference value between initial and final fresh weight (**Table 1**) compared to the water hyacinth treated by Cr. In water hyacinth, Cr can inhibit vegetative growth. There was no initial dry weight measurement to keep the plants alive for three weeks (time of treatment).

4. Discussion

The results of the analysis of $Cr3^+$ in water showed that the water hyacinth plant samples have a higher Cr content compared with water lettuce plant. The low $Cr3^+$ content in ppm for water lettuce plants, but still, there is no evidence for this mechanism especially for $Cr3^+$ absorption.

water lettuce water samples showed that the absorption of $Cr3^+$ by water lettuce is more effective than water hyacinth. $Cr3^+$ treatment of water lettuce showed growth differences that produce a lot of small vegetative plants (tillers). Another research showed that *P. stratiotes* was able to efficiently reduce $Cr6^+$ in 16 days, thus this plant has a good performance in reducing $Cr6^+$ [20].

As shown in **Figure 1** and **2**, $Cr3^+$ content on the roots of water lettuce is higher than water hyacinth. For both water hyacinth and water lettuce, $Cr3^+$ content from the control treatment up to 80 ppm was increased but started to decrease at 120 ppm concentration. This shows the maximum ability of Cr absorption was likely to reach a concentration of 80

https://doi.org/10.5614/3bio.2020.2.1.2

Table 1. Plant biomass on different treatments

Weight (gram)	Water Hyacinth				Water Lettuce			
	Control	40 ppm	80 ppm	120 ppm	Control	40 ppm	80 ppm	120 ppm
Initial Weight	170	225	207	188	174	143	113	106
Final Weight	410	421	427	302	231	274	182	255
Dry weight	15.38	13.14	12.14	4.62	8.48	8.77	5.20	7.43

A previous study on *P. stratiotes* treated with $Cr6^+$ showed that the plant could effectively remove $Cr6^+$ from the solution with minor damage up to an initial $Cr6^+$ concentration of 3 mg/L, for which the adsorption isotherm studies were conducted. The maximum uptake capacity of the plant was recorded was 0.05 mg/g of Cr (VI) at the equilibrium level after a contact period of 7 days for an initial concentration of 8 mg /L, although severe physiological damage occurred [21].

The results of Cr analysis on the leaves of water lettuce are higher than those of water hyacinth. In the analysis of water hyacinth leaves, the higher Cr concentration coincides with the higher the Cr leaf content. However, on water lettuce, the control treatment up to 80 ppm experienced an increase of Cr concentration and at a concentration of 120 ppm, it decreased. In Figure 1 and 2, the concentration of Cr in water lettuce is higher than water hyacinth, although, at the concentration of 0 ppm the Cr content is slightly higher in water hyacinth, it is related to the mobility of heavy metals in plants. Each species has its own mechanism for stress, including heavy metal stress. Plasma membranes act as a barrier for heavy metal ions to be able to enter cells, so this plasma membrane plays an important position in plants that are resistant to heavy metals [22]. In water lettuce, although the absorption and mobility of Cr is high in the body, it affected the growth and increased mortality rate. Water hyacinth, however, can last longer in Cr contaminated water because the levels of Cr absorbed and the mobility of Cr are low in the body.

Based on this, water lettuce can be used for phytoremediation purposes in Cr polluted environments. Phytoremediation is one solution that is inexpensive, sustainable, and saves energy in contaminated areas. Phytoremediation is the application of plants for in-situ or ex-situ treatment/removal of contaminated soils, sediments and water [23]. One example of phytoremediation is phytostabilization [24]. Phytostabilization is an attempt to reduce the pollutant content in which plants are used as an agent to reduce heavy metal movement in the soil. Based on the results of the Cr test, the higher increasing of Cr level in the water, the higher the level Cr in plant tissue. The Cr plant tissue increased along with the increase of Cr concentration in water. This is because the concentration of Cr solution is more concentrated than in the root tissue, so Cr enters the root [25].

According to research in [25], the roots accumulate 10-100 times more than the shoots and other tissues. Several studies show that in general, the absorption capacity of heavy metals in plants followed a sequence: roots, stems, and leaves [26]. Cr toxic effects can be seen from plant morphology. The leaves became yellow, some almost come off the trunk, and some fall out. Other toxic effects also occur in the roots where some of the root fibers are separated from the roots. Cr in high concentrations also inhibits enzyme activity, damages root cells, and causes ultrastructural modifications to chloroplast and cell membranes [27]. This is consistent with the observations shown in Figure 5.

The Cr absorption is higher in water lettuce than water hyacinth. The difference in absorption is influenced by the characteristics of water plants. Water hyacinth can bind organic materials from mud particles which make this plant suitable to purify water. This plant also serves an ecological function as the water stabilizer due to its ability to neutralize pollutants in the waters [28]. Through its thick roots, Cr metal is absorbed and then used in metabolic processes or stored in roots, stems, tubers or leaves; and can absorb excess nutrients in the water. Water lettuce is very resistant to the low nutrient levels in the water but its response as well to high nutrient levels by forming a lot of vegetative tillers [28]. This is what causes the water lettuce to be more resistant compared to water hyacinth. Water hyacinth could absorb Cr as well as water lettuce but could not form a lot of tillers. Cr affect water uptake of plants by disrupting growth and development including changes in the germination process followed by changes in root, stem and leaf growth.

Several studies reported the plants' response to stresses that affect chlorophyll levels, such as drought stress. According to [29], chlorophyll concentration can be used as an indicator of the lack of water in plants. **Figure 4** shows that at concentrations of 0 to 80 ppm the chlorophyll content in water hyacinth was above the chlorophyll content of water lettuce; while at 120 ppm Cr concentration, the chlorophyll content of water lettuce was slightly higher. From the statistical test, it was found that there were no significant differences between the chlorophyll levels of the two plants for each different Cr concentration ($P > 0.05$). Heavy metals affect photosynthesis by influencing pigment formation, electron transport activity, and calvin cycle enzymes. Cr plays a role in inhibiting thylakoid formation in chloroplasts

https://doi.org/10.5614/3bio.2020.2.1.2

[30]. Besides, the main target of heavy metals is in the electron transport process. Although there was no significant difference in water hyacinth which was treated with the addition of Cr, chlorosis can be seen morphologically, i.e., a condition in which plant tissue fails to form chloroplasts resulting in pale green or yellowish leaf color. In this study the differences seen from the color of water hyacinth leaves as shown in **Figure 5. Figure 5** showed the chlorosis in leaves of water hyacinth and for water lettuce the chlorosis was not seen.

This research showed that there was a decrease in proline levels in water hyacinth plants along with the increase in the concentration of Cr; however, there were no statistically significant differences. A research by [31] on the effect of Cr on photosynthetic pigments, stress defense systems, nitrate reduction and proline levels on *Ocinum tenuiflorum* plants showed that the higher the concentration of Cr, the higher the level of proline, which was thought to be a form of defense against heavy metal stress. In this study, there was a decrease in proline levels with the increase in Cr, which can occur due to the administration of $Cr3^+$ with low toxicity [3], therefore, water hyacinth plants do not respond to $Cr3^+$ as a metal compound that causes stress.

In contrast to the response of water hyacinth, the proline levels in water lettuce showed that a low proline leveil in control and 40 ppm. However, the proline levels were quite high at 80 ppm concentration decreased at a concentration of 120 ppm. Several studies on plants responses to the changes in water content (drought, salinity, freezing) showed the formation of proline was one way to defend the plant [32]. Concerning heavy metal stress, Cr in high concentrations interferes with the process of absorption of water and nutrients by roots, so plants are not able to absorb water optimally and a process occurs that causes the plant to dry out and in response produces proline compounds.

The activity of catalase (CAT) enzyme in water hyacinth did not differed significantly ($p > 0.05$) in each treatment. For water lettuce, there was a significant difference ($p < 0.05$) on the addition of CrCl3 between a concentration of 80 ppm and 120 ppm with the control. Based on **Figure 5**, both water lettuce and water hyacinth experienced a decrease in CAT along with the increase in Cr (III) metal in water media. This is different from the results obtained by [33] who observed an increasing CAT with variations in Cr metal concentration from 0 to 5 mM. Another study by [34] showed a decreasing catalase enzyme activity in water hyacinth exposed to Cd between 50 and 75 ppm. CAT measurement is one of the groups of antioxidant enzymes that are commonly analyzed in plants exposed to heavy metals. These enzymes are peroxidase (POD) and superoxide dismutase (SOD). These tenzymes have the highest activity on plant roots, because roots are the initial entry of heavy metals into plant tissue systems [35]. The reason CAT decreased along with the increase of $Cr3^+$ was due to the possibility of not forming H_2O_2. Increase in H_2O_2 production were reported to occur during a biotic and/or abiotic stress regime, such as pathogen attack, wounding, UV irradiation, exposure to intense light, drought, salinity, and/or chilling [36].

The activity of catalase hyacinth and water lettuce enzymes were shown in **Figure 6**. Plants can avoid the entry of heavy metal ions into their bodies based on immobilization in the cell wall and then release cell ligands that act as chelating agents for metals. As a result, plants are able to make heavy metals that will enter as a non-toxic substance in intracellular processes [24].

Table 1 showed a change in the amount of biomass in each treatment both for water hyacinth and water lettuce. In water hyacinth, samples from the control treatment have the biggest weight difference between initial fresh weight and final fresh weight (240 gr); and for Cr3+ treatment 120 ppm, the weight difference was 114 gr, which shows that in water hyacinth, Cr^{3+} may inhibit vegetative growth.

In water lettuce, different results were obtained, the highest difference between initial fresh weight and final fresh weight was obtained from water lettuce with the 120 ppm Cr3+ concentration. Water lettuce is capable of producing vegetative tillers, and the number of vegetative tillers was increased in the treatment of 120 ppm Cr. Although the main plants are not able to live long in high Cr concentrations, but vegetative tillers can absorb Cr by suppressing their body size, resulting in much smaller tillers.

5. Conclusions

Both plants accumulated Cr metal in roots with the largest concentration of 1660 mg / Kg in *P. stratiotes* and 894.126 mg /Kg in *E. crassipes*, respectively, with both at 80 ppm treatment. It was found that there was no significant difference ($p > 0.05$) on chlorophyll and proline levels in each treatment for each species of aquatic plant. The activity of catalase enzyme decreased with increasing concentration of Cr^{3+} in water media, with a significant difference ($P < 0.05$) for *P. stratiotes* which was exposed to 80 to 120 ppm compared to the control and from this research water lettuce has better performance for trivalent chromium removal in water.

References

[1] Owlad M, M.K. Aroua, W.A.W. Daud, S. Baroutian. 2008. Removal of hexavalent chromium-contaminated water and wastewater: A review. Water Air Soil Pollute, 200:59-77.

[2] Di Palma, L., 2015. Hexavalent Chromium Reduction in Contaminated Soil: A Comparison Between Ferrous Sulphate and Nanoscale Zero-Valent Iron. J. Hazard. Mater. 281: 70-76.

[3] Xu B., Fang W., Qiuhong Z., Qingqing L., Chaoyang L., Xuan G., Qiuying C., Yanhui C., Guo W., Jing D., 2018. Influence of iron Plaque on the Uptake and Accumulation of Chromium by Oryza sativa L. seedlings: Insight from Hydroponic and

https://doi.org/10.5614/3bio.2020.2.1.2

Soil cultivation. Ecotoxicology and Environmental Safety, 51-58.

[4] Singh, H.P. 2013. Chromium Toxicity and Tolerance in Plants. Environ. Chem. Lett 11, 229-254.

[5] Yadav, S.K. 2010. Heavy Metal Toxicity in Plants: An Overview on the Role of Glutathione and Phytochelatin in Heavy Metal Stress Tolerance of Plants. South African Journal of Botany,167-179.

[6] Tanner, R. 2017. Pistia stratiotes L. Bulletin OEPP/ EPPO Bulletin 47 (3), 537-534.

[7] Cervantes, C., Campos-Garcia, J., Devars, S., Guiterresz-Corona, F., Loza-Tavera, H., Torres-Guzman, J.C., Moreno-Sanchez, R. 2001. Interactions of Chromium with Microorganisms and Plants. FEMS Microbiology Reviews 25, 335-347.

[8] Shanker A.K., Cervantesb C., Loza-Taverac H., Avudainayagam, 2005. Chromium Toxicity in Plants. Environmental International 31 , 739-753.

[9] Vajpayee, P., Tripathi, R.D., Rai, U.N., Ali, M.B., Singh, S.N. 2000 Chromium Accumulation Reduces Chlorophyll Biosynthesis, Nitrate Reductase Activity and Protein Content in Nympaea alba L. Chemosphere 41, 1075–1082.

[10] Skeffington, R.A., Shewry, P.R., Perterson, P.J., 1976. Chromium Uptake and Transport in Barley Seedlings (Hordeum vulgare L.). Planta 19, 807–810.

[11] Viktor, K., Ladji, M., Adjiri, A., Cyrille, Y. D. A., Sanogo, T. A. Bioaccumulation of Heavy Metals from Wastewaters (Pb, Zn, Cd, Cu and Cr) in Water Hyacinth (Eichhornia crassipes) and Water Lettuce (Pistia stratiotes). International Journal of ChemTech Research Vol.9. 2016. No.02 , 189-195.

[12] Asati, A., Pichhode, M., Nikhil, K. 2015. Effect of Heavy Metals on Plants: An Overview. IJAIEM .ISSN 2319- 4847.

[13] Shanker AK, Sudhagar R, Pathmanabhan G. 2003. Growth Phytochelatin SH and antioxidative response of Sunflower as affected by chromium speciation. In: 2nd international congress of plant physiology on sustainable plant productivity under changing environment, New Delhi.

[14] Kalra, Yash. P. 1998. Handbook of Reference Methods for Plant Analysis. Ch 24. CRC Press. New York.

[15] MacFarlane, G.R., E.C. Koller, and S.P. Blomberg. 2007. Accumulation and Patitioning of Heavy Metals in Mangrove: A Synthesis of Field-based Studies. Chemosphere, 1454-1464.

[16] Yoon, J., C. Xinde, Z. Qixing , and L.Q. Ma. 2006. Accumulation of Pb, Cu, and Zn in Native Plants Growing on a Contaminated Florida Site. Science of the Total Environment, 456-464.

[17] Hendry, G.A.F. dan Grime, J.P. 1993. Methods in comparative plant ecology. Springer, New York.

[18] Bates, L.S. Waldren, R.P. Teare, I.D. 1972. Rapid Determination of Free Proline For Water Stress Studies. Plant & Soil 39, 205-207.

[19] Maksimovic, J.J.D dan Zianovic, B.D. 2012. Quantification of The Antioxidant Activity in Salt-Stress Tissue. 237-250

[20] Mayzarah, E, M., Moersidik, S. S., Saria, L. 2018. Control of Chromium Hexavalent (Cr -VI) Pollution on Waste Water in Nickel Ore Extraction Industry with Phytoremediation Technology. E3S Web of Conference 68, 03011.

[21] Chakraborty, R., Karmakar, S., Mukherjee, S., Kumar, S. 2014. Kinetic Evaluation of Chromium (VI) absorption by water lettuce (Pistia). Water Sci Technol69 (1): 195–201.

[22] Duca, M. 2015. Plant Physiology: Biological and Medical Physics, Biomedical Engineering. Springer.

[23] Aisien, F. A, Faleye, O., Aisien, E. T. 2010. Phytoremediation of Heavy Metals in Aqueous Solutions . Leonardo Journal of Sciences, 37-46

[24] Ma, L. Q., K.M. Komar, C. Tu, and W. A. Zhang. 2001. A fern that Hyperaccumulator arsenic. Nature , 409:579

[25] Isratul Izzah, Supriatno dan Wardiah. 2017. Kiambang (Pistia stratiotes) sebagai agen fitoremediasi logam krom (Cr). Prosiding Seminar Nasional Biotik.

[26] Yap, C.K., Fitri, Mohd, M. R., Mazyhar, Y. & TA, S.G. 2010. Effects of Metal Contaminated Soils on The Accumulation of Heavy Metals in Different Part of Centella asiatica. A Laboratory Study. Sains Malaysiana 39 (3).

[27] Panda, SK, Choudhury S. 2005. Chromium stress in plants. Brazilian Journal of Plant Physiology 17:95-102.

[28] Puspita, U.R., Siregar, A.S, Hidayati, N.V. 2011. Kemampuan Tumbuhan Air Sebagai Agen Fitoremediator Logam Berat Kromium (Cr) Yang Terdapat Pada Limbah Cair Industri Batik. Berkala Perikanan TERUBUK, 39 (1).

[29] Ai, Nio Song dan Banyo, Y. 2011. Konsentrasi Klorofil Daun Sebagai Indikator Kekurangan Air pada Tanaman. Jurnal Ilmiah Sains Vol 11. No 2, 166-173.

[30] Pandey, V., Dikshit V., Shyam, R. 2012. Hexavalent Chromium Induced Inhibition of Photosynthetic Electron Transport inIsolated Spinach Chloroplast. Intech, 229-244

[31] Rai, V and Mehrotra, S. 2004. Effect of Chromium Accumulation on Photosynthetic Pigments, Oxidative Stress Defense System, Nitrate Reduction, Proline Level and Eugenol Content of Ocimum tenuiforum L. Elsivier. Plant Science Vol.167: 1159-1169.

[32] Verslues, P.E and Sharma, S., 2010. The Arabidopsis Book: Proline metabolism and its Implication for Plant-environment Interaction. American Society of Plant Biologist. E0140.10.1199/tab0140

[33] Odjegba, V.J. and Fasidi, I.O. (2007). Changes in antioxidant enzyme activities in Eichhornia crassipes (Pontideriaceae) and Pistia stratiotes (Araceae) under heavy metal stress. Rev. Biol. Trop. (Int. J. Trop. Biol.). 55(3-4):815-823.

[34] Borker, A.R., Mane, A.V., Saratale, G.D., and Pathade, G.R. (2013). Phytoremediation potential of Eichhornia crassipes for the treatment of cadmium in relation with biochemical and water parameters. Emir. J. Food. Agric. 25(6) : 443-456. doi:10.9755/ejfa.v25i6.13970

[35] Sangya, S.B., Lawrence, K. and Pandey, A.K. 2016. Phytoremediation Potential of Eichhornia crassipes (Mart.) Solms. IJEAB. 1(2) : 210-217.

[36] Sofo, A., Scopa, A., Nuzzaci, M., Vitti, A. 2015. Ascorbate Peroxidase and Catalase Activities and Their Genetic Regulation in Plants Subjected to Drought and Salinity Stresses.Int. J. Mol. Sci. 16, 13561-13578 OPEN ACCESS

https://doi.org/10.5614/3bio.2020.2.1.2

e-ISSN: 2655-8777 https://journals.itb.ac.id/index.php/3bio

Chemical content in two Teak woods (*Tectona grandis* Linn.F.) that has been used for 2 and 60 years

Enih Rosamah[1]*, Fera Ferliyanti[1], Harlinda Kuspradini[1], Rudi Dungani[2], Pingkan Aditiawati[2]

1) Faculty of Forestry, Mulawarman University, Samarinda, Indonesia
2) School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*Corresponding Author: enihros@gmail.com
Received 05/12/2019
Accepted for publication 21/04/2020

Abstract

Teak (*Tectona grandis* Linn F.) is classified as luxury wood and belongs to the durable wood, resistant to termite and fungal attacks. The purposes of this study were to analyze and compare the chemical content and bioactive compound of teak (*T. grandis* Linn F.) from Sumedang, West Java based on age of use. This study used teak woods of 2 and 60 years of use. The chemical components were analyzed by determination of lignin, extractives, and ash content. Phytochemical compounds were analyzed by changes in color of crude acetone extracts. The results showed that the teak wood used for 2 years contained i.e., 28.41% lignin; 4.26% cold water soluble extractives; 5.12% hot water soluble extract; 19.4% NaOH (1%) soluble extractives; 6.21% alcohol:benzene (1:2) soluble extract; and 0.85% while ash content. Meanwhile, teak wood used for 60 years contained, i.e., 29.82% lignin; 1.56% cold water extract; 2.56% hot water extract; 12.30% NaOH (1%) soluble extract; 4.62% alcohol:benzene (1:2) extract; and 1.36% ash content. The qualitative phytochemical test demonstrated both of teak wood used for 2 and 60 years contained flavonoids, tannins, triterpenoids, cumarins, and carbohydrates.

Keywords: teak wood, age of use, lignin, extractives, phytochemical, bioactive compounds

1. Introduction

Teak is one of the most popular wood species used since many decades ago for its characteristics, i.e., unique, elegant, stable, and easy to process. Teak is classified as fancy wood and durable class II which were resistant to termites and fungi [1]. Until now, teak is still considered a luxury commodity that received a great public demand, even though the selling price is expensive [2,3].

The efficiency of overall timber utilization in the hope of meeting the increasing demand for wood in Indonesia has been encouraged by several factors, i.e., the limited availability of the high-quality teak wood on the market for the last 5-10 years, the tendency of declining natural forest resources and plantations, and the increasing demand for wood. The efficient use of wood depends on how much knowledge of the wood is available. Every wood has different properties, as well as for similar woods with unequal usage times. Even wood from one tree has a somewhat different nature. In this case, it is better if the nature of wood is adjusted in its use as building materials, household furniture, wood processing, and wood energy. The intended characteristics are anatomical, physical, mechanical and chemical properties [4, 5]. bioaccumulator agent.

Many properties of wood have directly and indirectly related to wood properties and the architecture of its compilation at the macroscopic and microscopic levels. Chemical properties have a considerable influence on the general nature of wood [6]. In other words, the chemical composition of wood has an important meaning because it determines the use of a certain type of wood. These chemical components are the main constituent components of wood cell walls which consist of cellulose, hemicellulose, lignin, and its accompanying components or components of wood cell micromolecules, (i.e., extractive substances). Also, the chemical compounds of plants are the result of the plant metabolism themselves [5]. Several researches have shown that these chemical compounds often have physiological and pharmacological effects that are beneficial to humans [3]. These chemical compounds are better known as secondary metabolites which are the result of irregularities in the primary metabolites of plants. These compounds are groups of alkaloids, steroids, terpenoids, phenols, flavonoids, saponins, etc.

https://doi.org/10.5614/3bio.2020.2.1.3

Research on the wood chemical components and chemical compounds of after a certain period of use is still not widely known, especially for teak wood after a 2-year and 60 years of usage period. Therefore, it is important to know how much the wood samples differ according to their usage period.

The purpose of this study is to analyze and compare the chemical and phytochemicals components of teak (*T.grandis* Linn. F.) which grew in West Java based on usage period differences as one of the basic properties of wood. The results of this study are expected to be useful for stakeholders who need information about the levels of chemical and phytochemicals components of teak (*T. grandis* Linn. F.) based on differences in usage period.

2. Experimental design

2.1. Sample preparation

The teak wood used in this study was taken from a house building in Sumedang, West Java, where the wood was obtained from a local (family-owned) community teak plantation. The raw material preparation procedure was as follows: the wood material obtained is cleaned, cut into chips with a thickness of ± 2 cm, dried and put into a plastic bag clip, made into particles in the form of powder using a hammer mill and sieved using a 40-60 mesh size and then put into a plastic bag clip.

2.2. Wood chemical component analysis

The method used refers to the TAPPI standard, such as Moisture Factor measurement (TAPPI T-264 om-88) [7], extractive free analysis of the sample (TAPPI T-204 om-84) [8], lignin Classon testing (TAPPI T- 222 om-88) [9], the solubility of extractive substances in cold water (TAPPI T-207 om-88) [10], the solubility of extractive substances in hot water (TAPPI T-207 om-88) [10], solubility of extractive substances in 1% NaOH (TAPPI T-212 om-93) [11], solubility of extractive substances in Alcohol-benzene (1: 2) (TAPPI T-204 om-88) [8] and testing of ash content (TAPPI T-211 om-85) [12].

2.3. Phytochemical analysis

Before the test is carried out, the sample is previously extracted and concentrated. Phytochemical tests were done on bioactive compounds, i.e., alkaloids, flavonoids, flavonoids, saponins, tannins, triterpenoids and steroids, carotenoids, coumarin, and carbohydrates.

2.4. Chemical compound analysis

The gas chromatography-mass spectrum (GC-MS) determination of the chemical compound of the teak wood extractives was performed using a Shimadzu GC MS-QP2010 Ultra. An elastic quartz capillary column Rtx-5ms coated with a neutral phase was used. The injection port temperature was 300oC, and the carrier gas helium. The program of Mass Spectrometer (MS) was scanned over the 1.5 AMU to 1090 AMU (m/z), with an ionizing voltage of 70 eV and an ionization current of 150 μA of electron ionization [1].

3. Results and discussion

3.1. Teak chemical component determination

The chemical component determination test was conducted to compare the percentage of chemical component content in teak wood. The wood chemical components tested include lignin, the solubility of extractive substances in cold water, solubility of extractive substances in hot water, solubility of extractive substances in 1% NaOH, the solubility of extractive substances in al-ben (1: 2), and ash content. The value of the average content of wood chemical components is shown in **Figure 1**.

The results of the analysis of the chemical components indicate that teak wood used for 2 years (T2) has a lower lignin content (28.41%) compared to teak wood used for 60 years (T60; 29.82%). Extractive substance content of T2 was higher than T60 (**Figure 1**), i.e., 4.26% soluble in cold water, 5.12% soluble in hot water, 19.40% soluble in 1% NaOH, and 6.21% soluble in alcohol:benzene (1:2). The original compounds of T60 were volatilized [15]. While the ash content was higher in T60 (1.36%). One possible explanation is that a high lignin content of wood with a high proportion of guiasyl monomers will be more condensed, therefore it will increase the density and hardness of wood [13].

The solubility of extractive substances in teak wood showed the lowest value in the solubility of cold water and the largest in NaOH 1%. Coldwater only dissolves substances that exist on the outside such as dyes, tannins, and less carbohydrates. The components of extractive substances that are soluble in cold water, i.e., glucose, fructose, carbohydrates, sugars, pectins, dyestuffs, and certain acids [14]. The extraction using cold water will produce components i.e., inorganic salts, gums, ingredients that resemble pectin, galactans, tannins, and pigments. Extractive substances that dissolve in hot water includes fats, dyestuffs, tannins, resin, and phlobatanin. The Alben-soluble extractive substances include tannins, phlobatanin, essential oils, dyes, resins, fats, fatty acids, waxes, gums, and some water-soluble substances. The content of extractive substances which are soluble in 1% NaOH are fatty acids consisting of fatty acids, waxes, resins, resin acids, sterols, unsaturated fatty acids, oleic acid, and linoleic acid. NaOH also dissolves most of the hemicellulose, especially its branch chains from pentose, hexose and organic acids.

https://doi.org/10.5614/3bio.2020.2.1.3

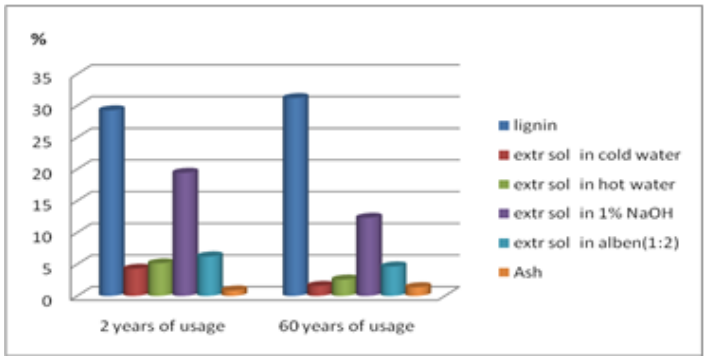


Figure 1 Chemical components of Teak wood of 2 and 60 years of use

Extractive substances generally consist of a group of volatile compounds. The longer wood being used and exposed to air, the more extractive compounds will be evaporated into the air. Therefore, the extractives content decrease in the wood [15].

The ash content of T60 is higher compared to T2. The determination of ash content is a way of estimating the mineral content of food material. The ash contains salts or oxides of K, P, Na, Mg, Ca, Fe, Mn and Cu; also very small molecules such as Al, Ba, Sr, Pb, Li, Ag, Ti, As and others. Moreover, there are still some inorganic substances in the wood called parts of ash, i.e., ash-forming minerals that are left behind after lignin and cellulose are burned. This mineral content varies between 0.2-1% content based on the weight of wood.

3.2. Phytochemical analysis

3.2.1. Extraction

Extraction was carried out using a maceration method with acetone solvent to extract the active compound in wood samples. The sample powder was soaked for 2 × 24 hours then concentrated to obtain a crude extract. The weight and yield of extracts from two types of samples is shown in **Table 1**.

The data presented in **Table 1** showed that the highest yield of 1.95% was found in T2 and the smallest yield of 1.85% was found in T60.

3.2.2. Phytochemicals

The phytochemical test was done to identify the plant active compounds content. In this study, the test was carried out by taking a small sample of the maceration extract and adding the reagent according to the identified compound. Qualitative phytochemical tests on T2 and T60 showed the positive results of flavonoids, tannins, triterpenoids, coumarin, and carbohydrates compound. While alkaloids, saponins, steroids, and carotenoids showed negative results. Test results can be seen in **Table 2**.

Some studies indicated the extractives function was responsible for wood durability, antioxidants, and protect the wood against photodegradation. The plant chemical compounds that refer to secondary metabolites play a role as a protector against pests or other disturbances and also have bioactivity. In economic terms, secondary metabolites can be used as antimicrobials, stimulants, toxicity, attractants, plant breeding, allelopathic effects, and physiological stress responses [16].

3.3. Analysis of chemical compound with GC-MS

Analysis of Teak wood extractives was done to identify the chemical compound that occurred in acetone soluble extractives [17]. The analysis was conducted using gas chromatography-mass spectrometry (GC-MS) due to some volatility extractives chemical compounds that can be elucidated by the mobile phase of GC-MS gas [18]. The results of GC-MS analysis is shown in **Table 3**.

The GC-MS chromatogram did not reveal the volatile compounds in T2. This means that a considerable part of the extractive substances in wood may be present in a macromolecular (insoluble) form or firmly bound to the skeleton component lignin or polysaccharides that it is not extractable by means of a neutral solvent. Another possibility, the greater part of the wood is made of polysaccharides. The major component is cellulose, which constitutes approximately one half of the wood substance.

On the other hand, GC-MS chromatogram reveals some the volatile compound in T60 as presented in **Table 4**.

https://doi.org/10.5614/3bio.2020.2.1.3

Table 1 Results of Teak wood maceration using acetone solvents

No	Time of usage	Initial weight (g)	MF	Extracts weight (g)	Yield (%)
1	2 years	50	0,9247	1.0572	1.9551
2	60 years	50	0,9213	1.0023	1.8468

Table 2 Phytochemical testing of Teak Wood (*Tectona grandis* Linn. F.) originated from Sumedang West Java with 2 and 60 years of usage

No	Phytochemical	Presence of compound	
		Teak wood after 2 years usage	Teak wood after 60 years usage
1.	Alkaloid	-	-
2.	Flavonoid	+	+
3.	Saponin	-	-
4.	Tannin	++	+
5.	Triterpenoid	++	+
6.	Steroid	-	-
7.	Carotenoid	-	-
8.	Coumarin	++	+
9.	Carbohydrate	+	++

Remark : (+) Identified compounds (++); strong, (+); weak. (-) Not identified compounds

Table 3 Analysis of extractives chemical compounds from teak wood after 2 years usage

Peak	R. Time	Area	Area%	Height	A/H	Name
2nd	1.375	14606	0.11	14554	1.00	Methanethiol (CAS) Merchaptomethane

Table 4 Analysis of extractives chemical compounds from teak wood after 60 years usage

Peak	R. Time	Area	Area%	Height	A/H	Name
16th	22.209	2455133	9.78	887697	2.77	9,10-Anthracenedione, 2-methyl-(CAS) 2-Methylanthraquinone

The extractive components comprise an extraordinary diversity of compounds. The proportions exhibit wide variation and some of these components are found in significant quantities in only a few species or genera. Thus, wood is more definitely characterized by the nature and amounts of the extractives than by the proportions of the cell wall component.

T60 sample reveals some extractive components such as 2-methylanthraquinone (2-MeA) or tectochinon or else tectoquinone, and 1,3-Indandione, 2 phenyl. A representative GC-MS chromatogram of the 2-methylanthraquinone mass spectrum is shown in Fig. 2. The chromatographic peak of 2-MeA was detected at R.time 22.209, area 9.78%, height 887697.

For many years, 2-MeA has been well-known and mentioned often as a chemical compound found in teak extracts [5]. The extractive components comprise an extraordinary diversity of compounds. The proportions exhibit wide variation and some of these components are found in significant quantities in only a few species or genera. Thus, wood is more definitely characterized by the nature and amounts of the extractives than by the proportions of the cell wall component. The extraneous substance may be present in wood, residing largely in the cell cavities. These include the extractives which can be removed from wood with neutral solvents, and other extraneous materials such as tannins, acids, fats, oils, sugar, proteins and pectic substances and others [5].

https://doi.org/10.5614/3bio.2020.2.1.3

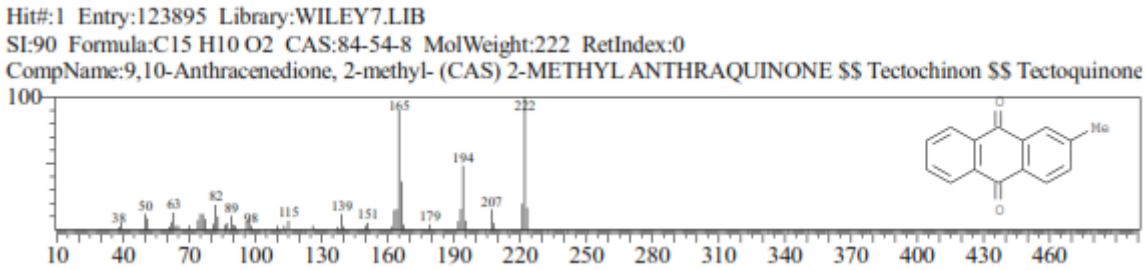


Figure 2 Extractives compound from teak wood after 60 years of use

4. Conclusions

The results of the analysis showed that the longer the wood is being used, the lignin content and ash content increase, while the extractive substance solubility decreases. The phytochemical analysis showed the longer the use of wood, the tannin, triterpenoid, and coumarin content decreased. The 2-Metylanthraquinone is a chemical component detected in teakwood of 60 years old of use.

Acknowledgments

The authors would like to thank The Ministry of Research, Technology, and Higher Education for providing the research grant for financial support through Program Dosen Merenung – DIKTI Project 2019. Grant Number: T/128/D2.3/KK.04.03/2019. September 27, 2019.

References

[1] Li, T., Li, G., Lu, Q., Zhou, J., Li, M., Zhang, S., Li, J. 2017. Characterization of *Tectona grandis* extractives by GC-MS and IR and their infusion into rubberwood to modify dimensional stability. Bioresources 12(3), 4500-4514.

[2] Dungani, R., Bhat, I.H., Abdul Khalil, H.P.S., Naif, A., Hermawan, D. 2012. Evaluation of antitermitic activity of different extracts obtained from Indonesian teakwood (Tectona grandis L.f). Bioresources 7(2), 1452-1461.

[3] Brocco, V.F., Paes, J.B., da Costa, L.G., Brazolin, S. 2015. Potential of teak heartwood extractives as a natural preservative against Nasutitermes corniger termite. The International Research Group on Wood Protection, the 46th IRG Annual Meeting Viña del Mar, Chile 10-14 May 2015.

[4] Rizanti, D.E., Darmawan, W., George, B., Merlin, A., Dumarcay, S., Chapuis, H., Gérardin, C., Gelhaye, E., Raharivelomanana, P., Sari, R.K., Syafii, W., Mohamed, R., Gerardin, P. 2018. Comparison of teak wood properties according to forest management:short versus long rotation. Annals of Forest Science 75(39), 1-12.

[5] Aung, U.M. 1988. Preliminary study on anthraquinone extractives in teak. Leaflet No. 8/87-88, 1-33.

[6] Lukmandaru, G. 2011. Variability in the natural termite resistance of plantation teak wood and its relations with wood extractive content and color properties. Indonesian Journal of Forestry Research 8(1), 17-31.

[7] TAPPI T-264 CM-97. 1997. Peparation of Wood for Chemical Analysis. Technical Association of the Pulp and Paper Industry.

[8] TAPPI T-204 CM-97. 1997. Solvent extractives of wood and pulp. Technical Association of the Pulp and Paper Industry.

[9] TAPPI T-222 CM-88. 2002. Acid-insoluble lignin in wood and pulp. Technical Association of the Pulp and Paper Industry.

[10] TAPPI T-207 CM-88. 1996. Water soluble extractive substance. Technical Association of the Pulp and Paper Industry.

[11] TAPPI T-212 CM-93. 2012. One percent sodium hydroxide solubility of wood and pulp. Technical Association of the Pulp and Paper Industry.

[12] TAPPI T-211 CM-02. 2012. Ash in wood, pulp, paper and paperboard: combustion at 525°C. Technical Association of the Pulp and Paper Industry.

[13] Haupt, M., Leithoff, H., Meier, D., Puls, J., Ricther, H.G., Faix, O. 2003. Heartwood extractives and natural durability of plantation-grown teakwood (Tectona grandis L .)-a case study. European Journal of Wood and Wood Products 61, 473–474.

[14] Lukmandaru, G., Takahashi, K. 2009. Radial distribution of quinones in plantation teak (Tectona grandis L.f.). Annals of Forest Science 66(605), 1-9.

[15] Li, H., Lei, X., Wu, Y., Hongchang, L., Wen, X.G., Hu, Y. 2019. Study of the discoloration behaviour of teak wood (Tectona grandis Linn.Fil.) caused by stimulated sunlight. Wood Research 64(4), 625-636.

[16] Lukmandaru, G. 2015. Quinones distribution of teak wood grown in community forest. Jurnal Ilmu dan Teknologi Kayu Tropis 13(2), 193-204.

[17] Lukmandaru, G. 2013. The Natural Termite Resistance of Teak Wood Grown in Community Forest. Jurnal Ilmu dan Teknologi Kayu Tropis 11(2), 131-139.

[18] Mankowski, M.E., Boyd, B., Hassan, B., Kirker, G.T. 2016. GC-MS characterizations of termiticidal heartwood extractives from wood species utilized in Pakistan. The International Research Group on Wood Protection, the 47th IRG Annual MeetingLisbon, Portugal15-19 May 2016.

https://doi.org/10.5614/3bio.2020.2.1.3

e-ISSN: 2655-8777 https://journals.itb.ac.id/index.php/3bio

Bioconversion of Napier Grass Mixed with Soybean Curd Residue Producing Bioethanol through Simultaneous Saccharification and Fermentation in Solid State Culture

Taufikurahman[1]* and Jessica[1]

1) Department of Bioengineering, School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*Corresponding Author: taufik@sith.itb.ac.id
Received 22/12/2019
Accepted for publication 20/04/2020

Abstract

Napier grass is a promising lignocellulosic biomass for bioethanol production because of its high cellulose content and high annual productivity. Converting a lignocellulosic biomass into a bioethanol usually takes two steps which resulted in a long processing time and sometimes includes the utilization of hazardous chemicals. Simultaneous saccharification and fermentation in a solid-state culture using single bioconversion agent, *Neurospora sitophila* Shear, can reduce the overall processing time and also increase the yield of the products. The research is aimed to determine the optimum aerobic and micro-aerobic conditions that yields the highest enzyme activity and bioethanol concentration from the mixture of Napier grass and soybean curd residue. The saccharification and fermentation process was conducted in the laboratory using an incubator at 33ºC. The cellulase enzyme activity was calculated as FPAse. The highest activity achieved was 0.538 filter paper unit with the optimum mass ratio of Napier grass to soybean curd residue was 1:1 on the second day of cultivation period. The fermentation process was conducted aerobically for two days and then followed by six days of micro-aerobic fermentation, resulting in the highest bioethanol yield of 2.12% (w/w) at the end of the culture period. The optimum mass ratio was found to be 9:1. This study shows that Napier grass and simultaneous saccharification and fermentation method has a great potential for cellulase and bioethanol production, but further improvement on the micro-aerobic system is needed to maximize the bioethanol yield.

Keywords: Bioethanol, *Neurospora sitophila*, FPAse, Simultaneous Saccharification and Fermentation, Solid State Culture

1. Introduction

Indonesia's energy requirement increases every year while the domestic production keeps declining. In 2016, Indonesia's fossil fuel consumption was 48.5 million kiloliters and increased to 55.4 million kiloliters in 2017. This value was much higher compared to fossil fuel production in the same year which was only 26.2 million kiloliters [1,2]. The government took an alternative approach by importing fossil fuel to solve this problem. However the depletion of fossil fuel resources, climate change, and sustainability issues are some of the reasons why we need to focus more on renewable energy.

Bioethanol is one of the renewable energy sources that can help us overcome this problem. The utilization of bioethanol as additives for fossil fuel can cut down the need for importing fossil fuel from another country up to 25%. The utilization of corn-based ethanol produced from dry mills can reduce the greenhouse gasses emission from 34% up to 108% [3]. Bioethanol utilization can also increase the gasoline octane number from 88 to 91-95 without the addition of hazardous chemicals such as Tetraethyl Lead (TEL) or Methy-Tert-Butyl Ether (MTBE) [4].

The first generation of bioethanol is produced from corn or sugarcane. It has demonstrated several sustainability issues [5]. The second generation bioethanol is also known as lignocellulosic bioethanol. Lignocellulose, as the major component of the plant cell

https://doi.org/10.5614/3bio.2020.2.1.4

wall, is a carbon source that is abundantly available thus ensures the availability of bioethanol raw material supply. Lignocellulosic biomass comprises cellulose, hemicellulose, and lignin [6]. Cellulose and hemicellulose are polymers from simple sugars, such as hexose and pentose, which is used as the substrate for hydrolytic enzyme production and bioethanol fermentation process while lignin is the component that can inhibit the process [7]. Napier grass (*Pennisetum purpureum* S.) is one of the perennial grasses that has a great potential to be the raw material for bioethanol production. Napier grass has a high cellulose content (around 30-37%) and low lignin content (8-21%) [8]. Its annual productivity is also high and can reaach up to 78 tons dry weight [9]. It has an efficient photosynthetic activity and water consumption, which makes this grass does not need any intensive care or special treatments [10]. These are the reasons Napier grass is a perfect candidate to be cultivated as an energy crop.

Bioconversion process of cellulose and hemicellulose to produce bioethanol comprises of two major steps, which are the hydrolysis of cellulose and hemicellulose into simple sugars and the fermentation process of the simple sugars into bioethanol.Various ways have been tried to increase the efficiency of bioethanol production. One of the methods is integrating the saccharification and fermentation process into one single process, called simultaneous saccharification and fermentation (SSF). This process can increase the efficiency of bioethanol production by reducing the product inhibition effect and reducing the number of reactors needed so it will reduce the investment cost for large scale production [11]. *Neurospora sitophila* is a mesophilic mold that can secrete cellulase and hemicellulase with an immense enzyme activity and simultaneously converting sugars produced by the saccharification process into bioethanol on micro-aerobic condition [12].

Mold naturally lives in a humid environment with the surrounding moisture content less than 100% [13]. Large scale ethanol fermentation system usually uses the submerged fermentation system despite the natural habitat of the mold. The solid-state fermentation process has no flowing water in the system, thus offers a living condition that is more favorable for *Neurospora sitophila*. The solid-state fermentation to produce enzymes and bioethanol using mold as the bioconversion agent hopefully will increase product yield. The addition of soybean curd residue was intended to increase the nitrogen and sugar content of the fermentation substrate. Soybean residue has 17-18 kinds of amino acids that can support the growth of the *Neurospora sitophila* [14]. Besides nitrogen, carbohydrates, and amino acids, soybean curd residue also has a huge amount of micronutrients that are important for the growth of the mold. The addition of soybean curd residue has been proven to increase the activity of cellulase enzyme produced by *Neurospora siophila* with paddy stalk as the substrate [15]. W expect that the combination of Napier grass and soybean curd residue and the utilization of simultaneous saccharification and fermentation in a solid-state culture can increase the enzyme activity as well as the bioethanol production by *Neurospora sitophila*.

2. Materials and Methods

2.1. Microorganism

Microorganism used in this experiment was *Neurospora sitophila* which was obtained from the Microbiology Laboratory Culture Collection, School of Life Sciences and Technology, Bandung Institute of Technology. *Neurospora sitophila* was cultured on a potato dextrose agar slant and subcultured every two weeks. Culture stock was stored on the refrigerator at 4^{o}C.

2.2. Viability Curve Analysis of Neurospora sitophila

Fungal spore viability analysis was conducted using the Total Plate Count method [16]. Spores attached on agar slant were diluted into particular concentrations (10^{-1}, 10^{-2}, and 10^{-3} more dilute than the original stock solution) and were counted using a hemocytometer under a light microscope. All of the diluted spores were cultivated on the PDA on Petri dishes and incubated on room temperature for several days. The colony-forming unit was calculated using equation (1).

$$\frac{CFU}{ml} = \frac{(A\,x\,10^{-1})+(B\,x\,10^{-2})+(Cx10^{-3})}{3} \quad (1)$$

notes:

A = Colony number on 10^{-1} dilution solution
B = Colony number on 10^{-2} dilution solution
C = Colony number on 10^{-3} dilution solution

2.3. Napier Grass and Soybean Curd Residue

The vegetative part of Napier grass was collected from CV Bintang Tani Bogor, West Java and then cultivated inside the screen house located on Bandung Institute of Technology, Jatinangor, West Java. Cultivation was done for 3 months and the Napier grass was harvested and later dried to be processed further. Additional substrate, the soybean curd residue, was collected from Mr. Cikeruh tofu production plant in Jatinangor, Sumedang, West Java.

2.4. Napier Grass and Soybean Curd Residue Preparation

Three-month old Napier grass was cleaned with flowing tap water and dried with shade drying method on room

https://doi.org/10.5614/3bio.2020.2.1.4

temperature for three days. The moisture content of the dried grass was below 10% (w/w). The dried grass was chopped with blender and passed up through mesh with 0.500 mm and 0.125 mm sieve opening so the particle size of the substrate would be between 0.500 mm and 0.125 mm.

The Soybean curd residue (SCR) was cleaned and dried using an oven on 50ºC. The SCR was dried until the moisture content reached 10%. The SCR was then chopped with a blender and sieved through the sieves with 0.500 mm and 0.125 mm opening. The SCR powder was stored inside an airtight container to be processed further.

2.5. Lignocellulosic Compound Analysis

Lignocellulosic compound on the mixed substrate was analyzed using Chesson-Datta fractioning method [17]. The lignocellulosic content on the substrate can be determined using equations (2) to (6).

$$Hot\ water\ soluble = \frac{A-B}{A}\ x\ 100\% \quad (2)$$

$$Hemicellulose = \frac{B-C}{A}\ x\ 100\% \quad (3)$$

$$Cellulose = \frac{C-D}{A}\ x\ 100\% \quad (4)$$

$$Lignin = \frac{D-E}{A} x\ 100\% \quad (5)$$

$$Ash = \frac{E}{A}\ x\ 100\% \quad (6)$$

Notes:
A = initial mass sample (g)
B = Residual mass after water extraction (g)
C= Residual mass after H_2SO_4 extraction (g)
D= Residual mass after H_2SO_4 72% extraction (g)
E= Residual mass after ashing (g)

2.6. Total Kjeldahl Nitrogen Analysis

Total nitrogen from the substrates that will be used for the experiment was analyzed using total Kjeldahl method [18]. Nitrogen percentage was calculated using the equation (7).

$$\%\ Nitrogen\ = \frac{(ml\ acid\ standard - ml\ blank) x\ N\ from\ acid\ x\ 1{,}4007}{dry\ weight\ of\ sample} \quad (7)$$

2.7. Aerobic Cultivation of Neurospora sitophila and Bioethanol Production

The solid-state fermentation process was done in two steps as described by Rao *et al.* in 1983 and Dogaris *et al.* in 2009 [12,19]. This process was carried out in 250 ml Erlenmeyer flasks with 25% of working volume consisting of the substrate and 75% was the headspace. On the first step, about three grams of mixed substrate were pasteurized using a water bath on 80ºC for one hour and were replicated three times. After the sterilization, the moisture content of the substrate was adjusted using glucose (5 g/L), yeast extract (6.4 g/L), and *Neurospora* minimal medium with the concentration of every component as follow: KH_2PO_4 1g/L; NH_4NO_3 1g/L; $MgSO_4.7H_2O$ 0.5 g/L; $CaCl_2$ 0.1 g/L; NaCl 0.1 g/L; $ZnCl_2$ 2 mg/L; $FeCl_3$ 0.3 mg/L; $CuCl_2$ 0.1 mg/L; $NaMoO_4.2H_2O$ 0.02 mg/L; $MnCl_2$ 0.02 mg/L; and H_3BO_3 0.01 mg/L

The fermentation medium was added until the moisture content of the substrate reached 70-80%. One ml of heavy spore suspension of *Neurospora sitophila* (approximately 10^7 spores/ml) was inoculated into the substrate. The sample was incubated on 33ºC for 6 days. The substrate combination is listed in **Table 1**.

Table. 1 The mass ratio of Napier grass to Soybean Curd Residue used in this research

Napier grass (g)	SCR (g)	Ratio
3	0	3:0
1.5	1.5	1:1
2.7	0.3	9:1

The cellulose enzyme activity was analyzed every day for six days. After the highest enzyme activity of the aerobic process was determined, the process was conducted all over again from the beginning and after two days, the system would be switched from aerobic condition to a micro-aerobic condition.

2.8. Enzyme Extraction

Fermentation substrate was harvested, weighed, and extracted using the method stated by Dogaris *et al.* [12]. Substrate was weighed using an analytical mass balance, and after the addition of the 50mM buffer solution, samples were incubated using an orbital incubator shaker (the incubator operated at 250 RPM and 28ºC for 60 minutes) and followed by the separation of the enzyme from the substrate using a filter cloth and a micro-centrifuge at 4ºC and 12000g. After that, samples were stored in the refrigerator at 4ºC before use.

2.9. Enzyme Activity Analysis (FPA Method)

Cellulase enzyme activity was analyzed using the Filter Paper Assay (FPA) Method [20]. Samples were diluted and calculated using the equation (8). Dilution from the sample had to be made if the reducing sugar detected was above 2 mg glucose equivalent/ml. Dilution was made until the concentration of the reducing sugar detected was around 2 mg glucose equivalent/ml. The enzyme concentration that is needed to release 2 mg of glucose was added to the equation (8).

$$FPU = \frac{0{,}37}{[enzyme] needed\ to\ release\ 2\ mg\ of\ glucose}\ unit/ml \quad (8)$$

Absorbance was calculated using pure glucose as the standard. Standard curve was made using 10, 3.35, 2.5,1.65, and 1 mg glucose/0.5 ml. Filter paper used was the

https://doi.org/10.5614/3bio.2020.2.1.4

Whatman® ashless filter paper no. 1, while the reagents used were Rochelle salt (10%) and DNS reagent.

2.10. Reducing Sugar Analysis

Reducing sugar analysis was conducted using Dinitrosalicyclic Acid (DNS) method [21]. The reducing sugar was calculated as glucose equivalent with pure glucose as the reference standard. The concentration of glucose standard solutions used was as follow: 10, 3.35, 2.5, 1.65, and 1 mg glucose/0.5 ml.

2.11. Bioethanol Concentration Analysis

Ethanol was extracted from the fermentation substrate using distillation method as stated by Devrajan [22]. Substrate that had been fermented was mixed with hot water (50ºC) in a 1:1 mass ratio. The mixture of sample and water was incubated for 30 minutes in a static condition. Sample then was distilled, along with the distillate, was collected to be analyzed further.

The ethanol concentration was measured using the spectrophotometer method as describe by Crowell and Ough [23]. Around 30-35 ml distillate was mixed with 10 ml of dichromate reagent (34 g of potassium dichromate mixed with 325 concentrated sulphuric acid) and incubated at 60ºC for 20 minutes. After 20 minutes, the sample was cooled down to room temperature, and the absorbance was measured using UV-VIS spectrophotometer on 600 nm wavelength. Alcohol detected was a 10 times dilution of the original sample.

$$Yse = \frac{Ef-Ei}{Sf-Si} x\ 100\% \quad (9)$$

Ef= Ethanol final weight (g)
Ei= Ethanol initial weight (g)
Sf= Substrate final weight (g)
Si = Substrate initial weight (g)

3. Results and discussion

3.1. Lignocellulose Composition of Napier Grass

Napier grass used in this research was a three-month old crop. Energy content form the three-month old Napier grass was the highest compared to the older or younger age Napier grass [24]. The comparison of lignocellulosic compounds compared to other studies can be seen in **Table 2**.

3.2. The Effect of Aerobic Cultivation Period on Cellulase Enzyme Activity

Cellulase enzyme activity was measured using the Filter Paper Assay (FPA) method. ANOVA analysis did not show any significant difference in enzyme activity from different substrate combinations, but the difference in cellulose reduction was up to 27% from the control. The cellulase activity profile for different substrate combinations is presented in **Figure 1**.

The control substrate gave the lowest cellulose reduction compared to substrate AT1 and AT2. Control substrate had a 45% cellulose reduction while AT1 and AT2 substrate reached 55.47% and 71.72% respectively. High cellulose reduction for substrate AT2 showed that the cellulose enzyme secreted by *Neurospora sitophila* on AT2 substrate was better at hydrolyzing cellulose compared to the other substrate combinations. Better mold growth perhaps is one of the reasons why the enzyme activity on AT2 substrate was better than the other two substrate combinations. Protein analysis from the three substrate combinations showed that the AT2 substrate has the highest protein enhancement compared to the other substrates. The measurement of total crude protein is one of the indirect method for measuring fungal growth on a solid substrate [30,31]. Protein enrichment profile can be seen in **Figure 2** and the fungal growth on the second days of aerobic cultivation can be seen in **Figure 3** (a)-(c).

High fungal growth on the AT2 substrate could be caused by the high initial nitrogen and extractives content of the substrates. From the lignocellulosic analysis, AT2 has the highest extractives content, approximately 25% weight, while the AT1 and control substrate has 23% and 16% respectively. The extractives content is usually comprised of pectin and other soluble oligosaccharides [17]. Soluble oligosaccharide can be used as an additional nutrition for the mold on the early stage of its growth. These soluble simple sugars can enhance the growth of the fungi. The composition of the lignocellulosic biomass of each substrate can be seen in **Figure 4**.

Soybean Curd Residue (SCR) contains micronutrients, for example potassium, sodium, calcium, magnesium, iron, copper, manganese, and zinc, which are needed by the *Neurospora*. Calcium is known to be beneficial for *Neurospora* hyphal growth. Schmid and Harold's (1988) experiment proved the importance of calcium ion for *Neurospora* hyphal growth. The growth of *Neurospora* and its hyphae is higher as the concentration of calcium ion increases [32]. External calcium ion was needed to balance the calcium ion inside the cell whereas the internal calcium ion is very important for hyphal growth [33].

Table 2 Lignocellulosic fraction comparison

Research	Hot water	Hemicellulose	cellulose	Lignin (%)	ash (%)	others (%)

https://doi.org/10.5614/3bio.2020.2.1.4

	soluble content (%)	(%)	(%)			
This research	16.1 ± 5.8	33.78± 1.6	40.35 ± 6.7	8.64 ± 2.4	1.1 ± 0.2	-
Yasuda *et al.*, 2013 [25]	-	26.50	37.50	14.90	12.70	15.10
Montipo *et al.*, 2018 [26]	16.43	20.62	33.60	18.42	12.25	0.99
Sladen *et al.*, 1991[8]	-	20-31	30-37	8-21	-	-
Kataria *et al.*, 2016 [27]	-	28.44	38.98	19.26	2.17	11.15

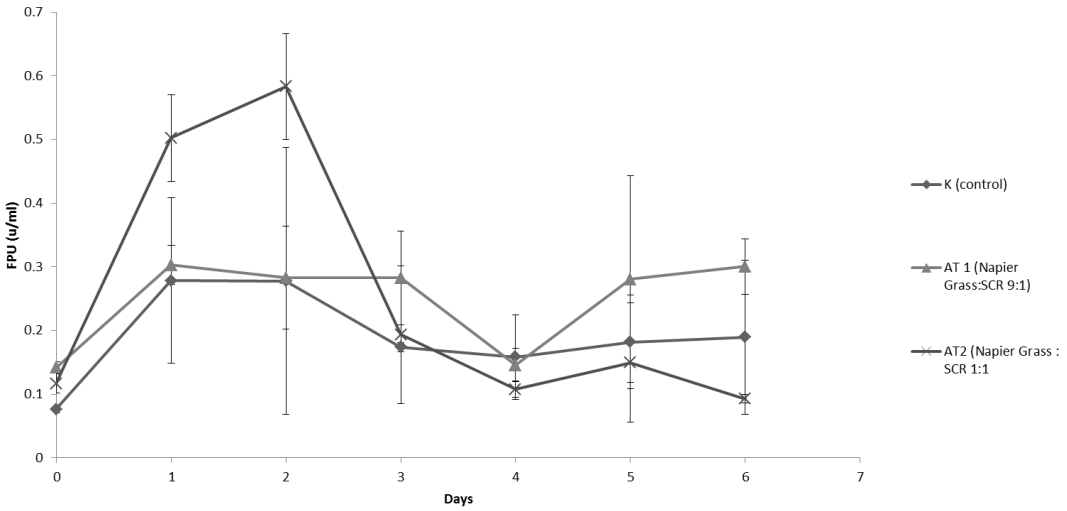


Figure 1 Enzyme activity profile for each substrate combination

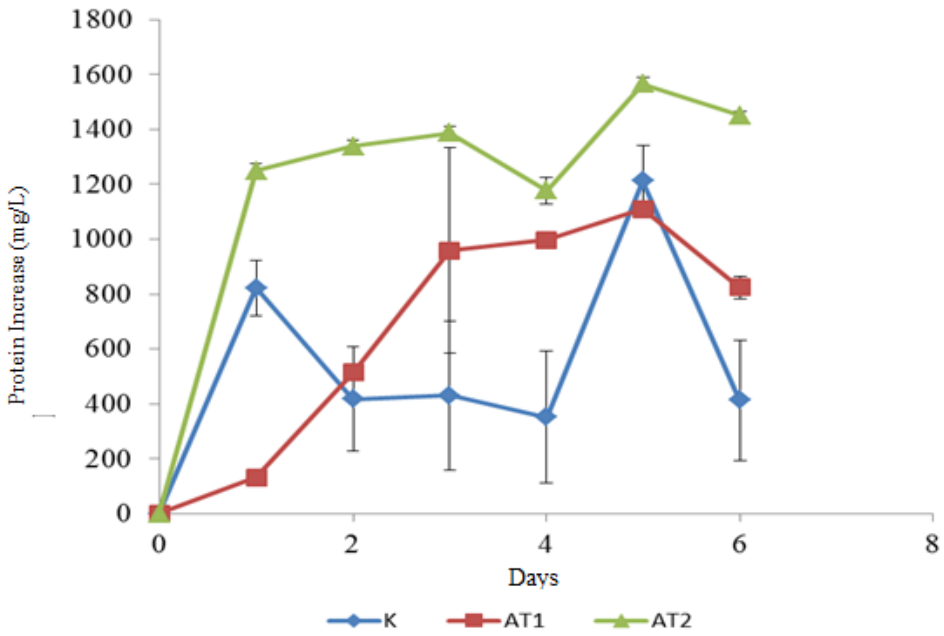
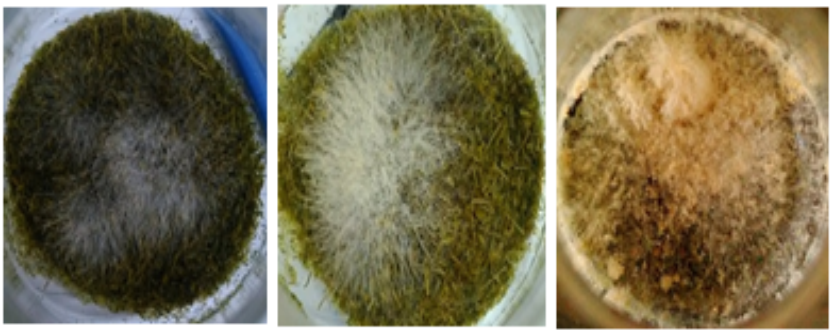


Figure 2 The increase of total protein content for each substrate combination

https://doi.org/10.5614/3bio.2020.2.1.4



a b c

Figure 3 The growth of *Neurospora sitophila* on each substrate combination on the 2nd day of aerobic cultivation period (a) Control, (b) AT1, (c) AT2

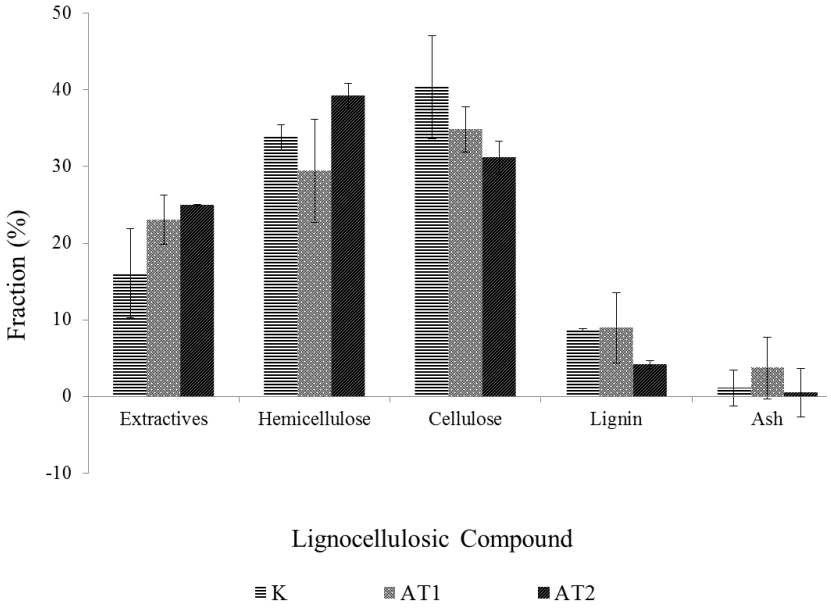


Figure 4 Lignocellulose fraction of fermentation substrate at the initial stage of cultivation

Neurospora sitophila has been known to be able to produce cellulase enzymes when cultivated in the submerged system as well as in the solid-state system [12,34,35]. According to Oguntimein *et al.* in 1991, cultivation of *Neurospora sitophila* in a submerged fermentation system can produce cellulase enzymes with the highest activity reached 0.18 FPU after 48 hours with a corn cob as the substrate [34]. Cellulase enzyme activity achieved on this research was higher compared to the study conducted by Oguntimein and his team. This comparison showed that solid-state fermentation system can produce a higher enzyme activity compared to the submerged system. This hypothesis was also proved by the study conducted by Kantini and Sudiana in 2018 [15]. Their research showed that cellulase enzyme produced by *Neurospora sitophila* on a solid state culture was given a better result compared to the *Neurospora* that was cultivated on a free -flowing medium.

One of the most possible reasons that the enzyme activity produced by *Neurospora* was better when it was cultivated on a solid substrate is because of the natural living conditions of the mold. Mold that was cultivated by submersion will have different physiological activities compared to the fungi that was cultivated on a solid substrate [14]. The use of solid-state fermentation system was intended to mimic the natural habitat of fungi, hence it can enhance its physiological activities relative to the submerged fermentation system [36]. Li and his team conducted a study in 2013 about the characteristic of the protein secreted by *Neurospora* on both solid-state and submerged fermentation system. On a submerged fermentation system, the protein analysis and

https://doi.org/10.5614/3bio.2020.2.1.4

characterization showed a large portion of proteins related to the fight and stress response of the fungi, while the protein profile for solid-state fermentation system was dominated by protein for growth [35].

Cellulase enzyme activity obtained from this study was higher compared to the study conducted by Li and his team. *Neurospora* cultivated on wheat stalks with a steam explosion as the pre-treatment gave cellulase enzyme activity of 0.45 U/gram substrate. Meanwhile, in this study, the highest FPAse achieved was 1.8 U/gram substrate from AT2 substrate combination (without a pretreatment before the fermentation process). The higher cellulase activity means that Napier grass is a better substrate compared to wheat stalks for cellulase production.

3.3. The Effect of Fermentation Period and Napier Grass-SCR Ratio on the Ethanol Yield

The highest ethanol yield from this study came from the AT2 sample (Napier grass : SCR mass ratio of 9:1). The ethanol yield from the AT2 sample was 2.12 g Ethanol/100 g substrate consumed. Ethanol yield from AT2 substrate and control substrate were 0.71 g ethanol/100 g substrate consumed and 1.09 g ethanol/g substrate consumed respectively. The comparison can be seen in **Table 3**.

Table. 3 Ethanol yield from each substrate combination

Substrate Combination	Ethanol Yield (g EtOH/100 g substrate)	Optimum Fermentation Period (days)
Control (K)	1.09	2
Napier Grass : SCR = 9:1 (AT1)	2.12	6
Napier Grass : SCR =1:1 (AT2)	0.71	3

From the data, it can be seen that the ethanol yield from the AT1 substrates was higher compared to the other substrates. It is assumed that there is a lower cellulose fraction of the AT2 substrate compared to the AT1 substrate. However, the cellulose fraction itself was probably not the only factor affecting the ethanol production. The ethanol yield from the control substrate was also lower than the AT1 substrate, despite its higher cellulose content. The better growth profile of *Neurospora sitophila* on the AT1 substrate was probably the main reason why the ethanol production from the AT1 substrate was higher than the control substrate.

From this study, it was found that the maximum ethanol concentration was achieved on the sixth day of the micro-aerobic cultivation period which was 1.874 g/L from the AT1 substrate. The AT2 substrate produces 0.811 g/L ethanol that was achieved on the fifth day of fermentation period. Meanwhile the control substrate achieved the highest ethanol concentration of 0.641 g/L on the 7th day of the fermentation period. From ANOVA test, it could be concluded that the difference of the ethanol concentration was significantly different for each substrate variation. The ethanol concentration profile was showed in **Figure 5**.

Despite the high cellulase activity secreted by *Neurospora* in this study, ethanol production was very low compared to other studies. Theoretical ethanol yield from this SSF process was calculated using the formula as shown in **Table 4** and the calculation for the three types of substrate combinations compared to the experiment based value is shown in **Table 5**. This formula and its conversion efficiencies were adapted from Badger [7].

The experimental based yield was approximately 10 times lower compared to the expected theoretical yield. The low ethanol yield was probably due to the excessive oxygen that was still present in the micro-aerobic process. In this study, the oxygen absorber used was an iron powder combined with NaCl salt that was sold commercially. The oxygen absorber can absorb around 30 ml of O_2. Using an assumption that the volume of the reactor used was 250 ml and the concentration of O_2 inside the reactor was the same as the concentration of O_2 in the normal air in the atmosphere, the volume of the oxygen in the headspace of 250 ml reactor was 50.4 ml. This calculation showed that there was still too much oxygen inside the reactor. The presence of oxygen inside the reactor made the fermentation process became ineffective. In a condition where the amount of oxygen is high, the dominant reaction will be the respiration pathway where the pyruvic acid will go into the tricarboxylic acid cycle and go through the electron transport chain reaction, thus resulting in ATP formation and carbon dioxide [33]. Purging the oxygen with nitrogen gas could result in a higher yield of ethanol [34].

During the fermentation process, it could be seen that the glucose concentration has never had any significant upsurge. This is due to the low glucose content on the first day of the micro-aerobic condition. When the cellulase enzyme hydrolyzed the cellulose, it produced reducing sugar which is simultaneously consumed by the fungi to generate biomass, ethanol, and other products. When the reducing sugar on the substrate is very low while the ethanol concentration is high, there will be a probability of alcohol dehydrogenase enzyme repression to occur and other enzymes that play role in glyoxylate cycle will be produced, thus resulting in ethanol consumption as a carbon source for the fungi via gluconeogenesis [35]. Glucose profile for each substrate combination is shown in **Figure 6** and the change in the lignocellulose compound during the fermentation process is shown in **Figure 7**.

From **Figure 7** (a)-(c), it can be seen that there were reductions both in cellulose fraction and hemicellulose fraction. This proved that *Neurospora sitophila* can produce both cellulase and hemicellulase enzymes. This conclusion was supported by the study conducted by Li and his team on

https://doi.org/10.5614/3bio.2020.2.1.4

2013 about the characterization of protein secreted by *Neurospora*, in which they also found out that *Neurospora* can secrete proteins that have similar amino acid sequences as the protein that encodes hemicellulose enzyme such as endo-1,4-ß-xylanase GH10-2 and ß-xylosidase GH3-8 [14]. *Neurospora* also has xylitol dehydrogenase enzyme that is a catalyst for the fungal cells to produce xylose derivate such as xylitol [37].

.

Table 4 Theoretical Ethanol Yield Formula

Parameters	Source of Carbohydrate	
	Hemicellulose	Cellulose
Dried Napier grass (gram) (DM)	3	3
Mass fraction (a)	% hemicellulose x DM	% cellulose x DM
Enzymatic conversion efficiency (b)	0.9	
Ethanol Stoichiometric yield (c)	0.51	
Fermentation efficiency (d)	0.50	
Ethanol yield (gram)	(a x b x c x d)/100 (P_1)	(a x b x c x d)/100 (P_2)
Total ethanol yield (gram/100 gram substrate)	P_1+P_2	

Table 5 Theoretical and Experiment Based Ethanol Yield for All Substrate Combinations

Substrate Variation	Ethanol Yield (g ethanol/ 100 g substrate)	
	Theoretical	Experiment Based
Control (K)	19.48	1.09
AT1	16.55	2.12
AT2	18.56	0.71

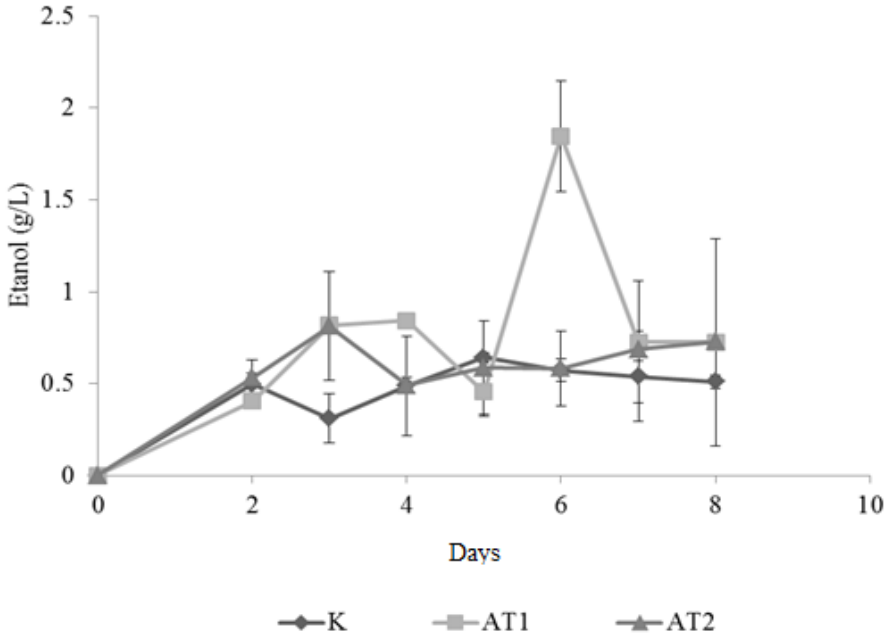


Figure 5 Ethanol Concentration Profile for Each Substrate Combination

https://doi.org/10.5614/3bio.2020.2.1.4

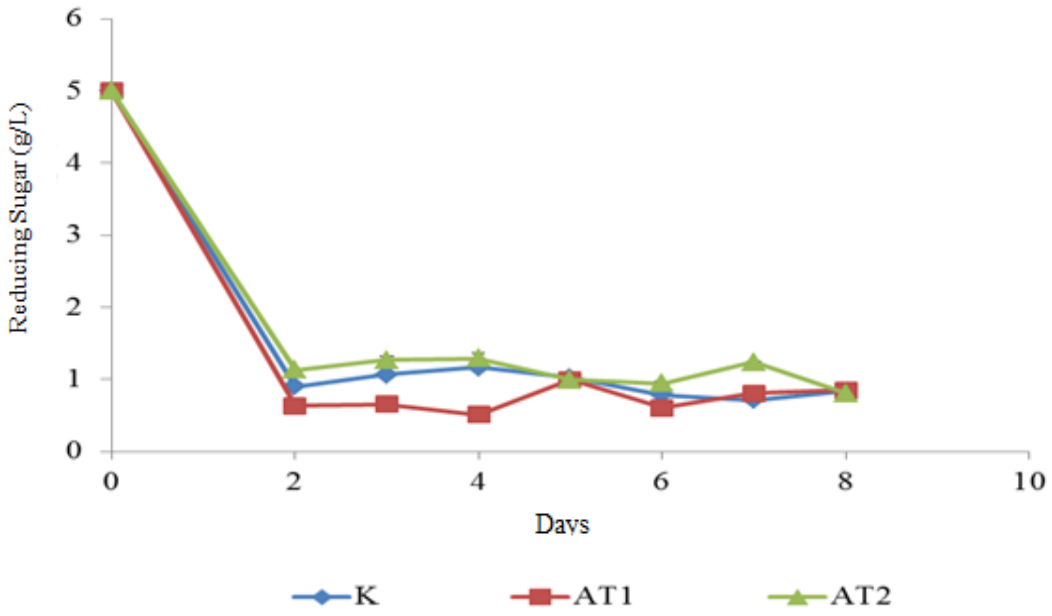


Figure 6 Glucose Reduction Profile for Each Substrate Combination

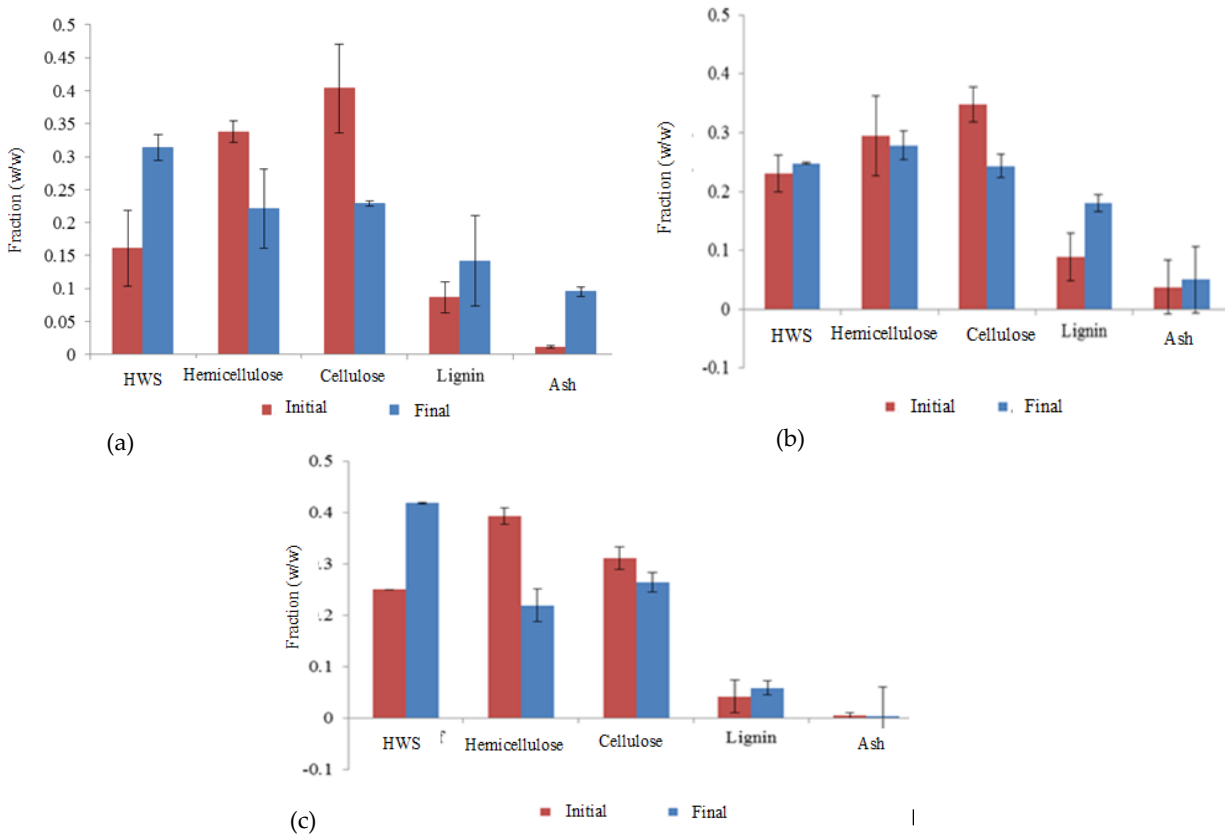


Figure 7 Hemicellulose reduction on the initial and final period of fermentation period
(a) Control Substrate (b) AT1 (c) AT2

https://doi.org/10.5614/3bio.2020.2.1.4

The extractives and lignin content from the substrate that had been fermented increased for every substrate combination. The ash content also increased for the control substrate. Napier grass has various compounds that were water soluble aside from free reducing sugar. Two of them were catechin and tannins. Catechin content inside Napier grass can be as high as 6 mg/g dry basis [38]. These compounds were stored inside a vacuole [39]. If the cellulose and hemicellulose content of the plant cell wall was reduced, these water soluble compounds could easily be washed out from the vacuole thus resulting in a higher fraction of hot water soluble content.

The increase of lignin fraction that oddly happened does not mean that the lignin compound of the substrate was increasing. The increase of lignin fraction on the substrate was suspected due to the decrease of other components fraction, whereby the lignin fraction will be higher because the fungi cannot consume it or it can be assumed that the lignin mass did not change.

4. Conclusions

In conclusion, Napier grass and simultaneous saccharification and fermentation method has a potential for cellulase and bioethanol production. The optimum aerobic cultivation period that gives the maximum cellulase enzyme activity was two days on the AT2 substrate. The optimum micro-aerobic cultivation period that gives the maximum ethanol yield was 6 days on the AT1 substrate combination. However, the bioethanol yields were still very low compared to the theoretical yields and other studies, thus further improvement on the micro-aerobic system is needed to increase the bioethanol yield.

Acknowledgment

We thank ITB for supporting this research through the P3MI project.

References

[1] BPH Migas 2017 Konsumsi BBM Nasional per Tahun.
[2] Duta D. 2016 Pertamina Impor BBM 8 Juta Barel per Bulan.
[3] US Department of Energy 2018 Ethanol Vehicle Emission. https://afdc.energy.gov/fuels/ethanol_benefits.html.
[4] Stradling R, Williams J, Hamje H and Rickeard D 2016, Effect of Octane on Performance, Energy Consumption and Emissions of Two Euro 4 Passenger Cars *Transp. Res. Procedia* **14** 3159–68
[5] Gasparatos A, Stromberg P and Takeuchi K 2013 Sustainability impacts of first-generation biofuels *Anim. Front.* **3** 12–26
[6] Rubin E M 2008 Genomics of cellulosic biofuels *Nature* **454** 841–5
[7] Badger P 2002 Ethanol from cellulose: A general review *Trends new Crop. new uses* 17–21
[8] Sladden S E, Bransby D I, Aiken G E and Prine G M 1991 Biomass yield and composition, and winter survival of tall grasses in Alabama *Biomass and Bioenergy* **1** 123–7
[9] Negawo A ., Teshome A, Kumar A, Hanson J and Jones C . 2017 Opportunities for Napier Grass (Pennisetum purpureum) Improvement Using Molecular Genetics *Agronomy* **7** 28
[10] Anderson W, Dien B, Brandon S and Peterson J 2008 Assesment of Bermuda and Bunch Grasses as Feedstock for Conversion to Ethanol *Appl.Biochem.Biotechnol* 13–21
[11] Olofsson K, Bertilsson M and Lidén G 2008 A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks *Biotechnol. Biofuels* **1** 7
[12] Dogaris I, Vakontios G, Kalogeris E, Mamma D and Kekos D 2009 Induction of cellulases and hemicellulases from Neurospora crassa under solid-state cultivation for bioconversion of sorghum bagasse into ethanol *Ind. Crops Prod.* **29** 404–11
[13] Manpreet S, Sachin S and Banerjee S 2005 Influence of process parameters on the production of metabolites in solid-state fermentation *Malalaysian Jounal Microbiol.* **1** 1–9
[14] Li S, Zhu D, Li K, Yang Y, Lei Z and Zhang Z 2013 Soybean Curd Residue: Composition, Utilization, and Related Limiting Factors *ISRN Ind. Eng.* **2013** 1–8
[15] Kanti A and Sudiana I M 2018 Production of Phytase, Amylase and Cellulase by Aspergillus, Rhizophus and Neurospora on Mixed Rice Straw Powder and Soybean Curd Residue *IOP Conf. Ser. Earth Environ. Sci.* **166**
[16] Wahyudi P 2008 Enkapsulasi Propagul Jamur Entomopatogen Beauveria bassiana Menggunakan Alginat dan Pati Jagung sebagai Produk Mikoinsektisida 53
[17] Datta R 1981 Acidogenic fermentation of corn stover *Biotechnol. Bioeng.* **23** 61–77
[18] Kjeldahl J 1883 A New Method for the Determination of Nitrogen in Organic Matter *Zeitschrift für Anal. Chemie* 366–82
[19] Rao M, Deshpande V, Keskar S and Srinivasan M C 1983 Cellulase and ethanol production from cellulose by Neurospora crassa *Enzyme Microb. Technol.* **5** 133–6
[20] Ghose T . . 1987 MEASUREMENT OF CELLULASE ACTIVITY *pure Appl. Chem.* **59**
[21] Miller G L 1959 Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar *Anal. Chem.* **31** 426–8
[22] Joshi V K and Devrajan A 2008 Ethanol recovery from solid state fermented apple pomace and evaluation of physico-chemical characteristics of the residue *Nat. Prod. Radiance* **7** 127–32
[23] Crowell E A and Ough C S 1979 A Modified Procedure for Alcohol Determination by Dichromate Oxidation *Am. J. Enol. Vitic.* **30** 61 LP – 63
[24] Rengsirikul K, Ishii Y, Kangvansaichol K, Sripichitt P, Punsuvon V, Vaithanomsat P, Nakamanee G and Tudsri S 2013 Biomass Yield, Chemical Composition and Potential Ethanol Yields of 8 Cultivars of Napiergrass (<i>Pennisetum purpureum</i> Schumach.) Harvested 3-Monthly in Central Thailand *J. Sustain. Bioenergy Syst.* **03** 107–12
[25] Yasuda M, Ishii Y and Ohta K 2015 Napier grass (Pennisetum purpureum Schumach) as raw material for bioethanol production: Pretreatment, saccharification, and fermentation *Biotechnol. Bioprocess Eng.* **19** 943–50
[26] Montipó S, Ballesteros I, Fontana R C, Liu S, Martins A F, Ballesteros M and Camassola M 2018 Integrated production of second generation ethanol and lactic acid from steam-exploded elephant grass *Bioresour. Technol.* **249** 1017–24
[27] Kataria R, Mol A, Schulten E and Happel A 2017 Bench scale steam explosion pretreatment of acid impregnated elephant grass biomass and its impacts on biomass

https://doi.org/10.5614/3bio.2020.2.1.4

composition, structure and hydrolysis *Ind. Crops Prod.* **106** 48–58
[28] Welker C, Balasubramanian vimal kumar, Petti C, Rai K, Debolt S and Mendu V 2015 Engineering Plant Biomass Lignin Content and Composition for Biofuels and Bioproducts *Energies* **2015** 7654–76
[29] Takara D and Khanal S K 2015 Characterizing compositional changes of Napier grass at different stages of growth for biofuel and biobased products potential. *Bioresour. Technol.* **188** 103–8
[30] Kikas T, Tutt M, Raud M, Alaru M, Lauk R and Olt J 2016 Basis of energy crop selection for biofuel production: Cellulose vs. lignin *Int. J. Green Energy* **13** 49–54
[31] Abd-Aziz S, Ang D C, Yusof H M, Karim M I A, Ariff A B, Uchiyama K and Shioya S 2001 Effect of C/N ratio and starch concentration on ethanol production from sago starch using recombinant yeast *World J. Microbiol. Biotechnol.* **17** 713–9
[32] Schmid J and Harold F M 1988 Dual Roles for Calcium Ions in Apical Growth of Neurospora crassa *Microbiology* **134** 2623–31
[33] Silverman-Gavrila L B and Lew R R 2000 Calcium and tip growth in Neurospora crassa *Protoplasma* **213** 203–17
[34] Oguntimein G, Vlach D and Moo-Young M 1992 Production of cellulolytic enzymes by Neurospora sitophila grown on cellulosic materials *Bioresour. Technol.* **39** 277–83
[35] Li Y, Peng X and Chen H 2013 Comparative characterization of proteins secreted by Neurospora sitophila in solid-state and submerged fermentation *J. Biosci. Bioeng.* **116** 493—498
[36] Holker U, Hofer M and Lenz J 2004 Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Appl. Microbiol. Biotechnol.* **64** 175–86
[37] Phadtare S U, Rawat U B and Rao M B 1997 Purification and characterisation of xylitol dehydrogenase from Neurospora crassa NCL communication No. 6347. *FEMS Microbiol. Lett.* **146** 79–83
[38] Tsai P-J, Wu S-C and Cheng Y-K 2008 Role of polyphenols in antioxidant capacity of Napier Grass from different growing seasons *Food Chem.* **106** 27–32
[39] SUZUKI T, YAMAZAKI N, SADA Y, OGUNI I and MORIYASU Y 2003 Tissue Distribution and Intracellular Localization of Catechins in Tea Leaves *Biosci. Biotechnol. Biochem.* **67** 2683–6

https://doi.org/10.5614/3bio.2020.2.1.4

e-ISSN: 2655-8777 https://journals.itb.ac.id/index.php/3bio

A Comparison of Alkali and Biological Pretreatment Methods in Napier Grass (*Pennisetum purpureum* Scumach.) for Reducing Lignin Content in the Bioethanol Production Process

Taufikurahman[1]*, Wendo Obert Delimanto[1]

[1)] Department of Bioengineering, School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*Corresponding Author: taufik@sith.itb.ac.id
Received 5/27/2019
Accepted for publication 4/20/2020

Abstract

Napier grass is one of lignocellulosic plants that has the potential to be converted as bioethanol due to high productivity and relatively fast harvesting time. However, the problems of processing lignocellulosic plants into bioethanol are the high lignin content and the different lignin structure of each plant. Lignin can inhibit biological agents in accessing cellulose and hemicellulose. Therefore, it is necessary to select and optimize the pretreatment process with the aim of degrading lignin and maintaining the value of the cellulose. This study was conducted to compare the effectiveness of two different pretreatments (biological and alkaline) in degrading lignin. For the alkali pretreatment, lime ($Ca(OH)_2$) was added to the Napier grass substrate using concentrations of 0.05, 0.1, and 0.5 grams/gram, which was then incubated at 23-25 ^oC each within 6, 24, and 96 hours period. For biological pretreatment, *Aspergillus niger* spore was used as an agent, which was incubated onto the Napier grass substrate using concentrations of 10^6, 10^7, and 10^8 cells/mL, an optimal temperature of 35^oC and within 1, 3, 5, 7, and 9 days period. As a comparison, *Phanerochaete chrysosporium* was also incubated using a concentration of 10^6 a temperature of 35 ^oC within 28 days period. The extracted Napier grass was then analyzed for lignocellulose content, which included hot water soluble, Hemicellulose, cellulose, lignin, and ash, using Chesson-Datta method and reducing sugar test. Comparison of biological pretreatment between *Aspergillus niger* and *Phanerochaete chrysosporium* showed that *Aspergillus niger* was better at degrading lignin, with a lignin-to-cellulose ratio of 24.3%, smaller than *Phanerochaete chrysosporium* at 30.645%. This ratio was furthermore compared with the ratio resulting from Alkali pretreatment, which showed that the former was proven to be more optimum.

Keywords: Lignin degradation, *Aspergillus niger*, *Phanerochaete chrysosporium*, alkali (Ca(OH)2), Napier grass

1. Introduction

The need for fuel (gasoline) in developing countries such as Indonesia is increasing in parallel with the technological developments, while petroleum energy reserves are decreasing on a daily basis. To resolve the need for fuel oil, the government has drafted an energy policy planned in Presidential Instruction No. 1 of 2006 and Presidential Regulation No. 5 of 2006, which stipulate the importance of alternative energy, especially biofuels. Biofuels in this context are defined as fuels in the form of solids, liquids and gases that are produced from organic materials [1].

One of the new renewable fuels/energy is obtained by converting biomass into bioethanol. Biomass is a term used to classify organic materials from plants or animals that are rich in energy reserves, hence the converted product is called bioenergy. Bioethanol is an alternative fuel that has several advantages compared to fuel oil. Bioethanol emits 19 to 25% lower CO gas when compared to fuel oil [2]. Bioethanol can also be produced from materials that contain lots of cellulose. Cellulose is found in agricultural and plantation waste. This resource has not been used optimally.

Bioethanol produced today is made from starch (corn) or sugar (sugar cane) which is basically a food commodity in

https://doi.org/10.5614/3bio.2020.2.1.5

Indonesia. Producing bioethanol from human food can cause the next problem which is the food crisis. Therefore, the production of second generation bioethanol using non-food biomass should be a major concern to be a future of an alternative energy.

One non-food ingredient that has the potential to supply bioethanol from cellulose is Napier grass (*Pennisetum purpureum* Schumach.). Napier grass can thrive in tropical environments such as the one in Indonesia. With a relatively shorter growing period (40-90 days), Napier grass shows the potential in meeting the availability of raw materials for producing up to 78 tons/ha/year of bioethanol, more than other similar grasses [3]. Aside from being an animal feed and companion plant, Napier grass has not been widely used.

Bioethanol production from cellulose materials requires several stages prior to fermentation. This is because according to Isroi (2008), cellulose materials consist of lignin-covered and hemicellulose-bound twisted fibers that are difficult to process [4]. One important step to break down lignin protection is by pretreatment. Lignocellulosic biomass cannot be hydrolyzed by enzymes without pretreatment, mainly because the lignin in plant cell walls forms four types of barriers to withstand enzyme activity [5].

This pretreatment aims to break down the lignin barrier, change the structure of lignocellulose, and make cellulose and / or hemicellulose more easily hydrolyzed [6]. Ideal pretreatment will reduce the lignin content and crystallinity of cellulose and increase the surface area for enzyme activity [7].

Pretreatment can be done with physical, chemical, biological, or even a combination of these methods. However, amongst the other type of pretreatments, Biological pretreatment is claimed to be the most efficient and eco-friendly. Biological pretreatment is a promising alternative because biological agents commonly have lignolytic enzymes capable of breaking down complex lignin structures. Biological pretreatment is also a sustainable method, has a high energy efficiency (because it does not require large energy), and also cost efficient. Biological pretreatment has the advantage of a physiochemical approach to processes that can occur under normal conditions and do not produce by-products that cannot be tolerated by the environment (hence, eco-friendly) [8]. Despite these major advantages, the use of biological pretreatment on a large scale of production is limited by several barriers, namely the reaction rate is generally very slow and requires a long time [8]. Therefore, research is needed to optimize the concentration and time of biological pretreatment of chemical pretreatment to get the best pretreatment method in increasing the accessibility of enzymes to cellulose.

2. Materials and Research

2.1. Materials Used

The materials used include PDA (Potato Dextrose Agar) medium, PDB (Potato Dextrose Broth) medium, distilled water, filter paper, fatty cotton, 18M concentrated sulfuric acid, acetic acid, spiritus, falcon tube, 96% alcohol, Tween80, NaCl, fungi medium, DNS reagent, distilled water, NaOH solution, HCl solution, and Solid $Ca(OH)_2$ [9][14].

2.2. Pure culture of Aspergillus niger and Phanerochaete chrysosporium

Pure culture of *Aspergillus niger* and *Phanerochaete chrysosporium* fungi were obtained from the Microbiology Laboratory, School of Life Science and Technology, Bandung Institute of Technology - Ganesha Campus in a test tube containing tilted agar (PDA). The pure culture was then stored in a refrigerator at around 4 ºC to maintain viability.

2.3. Napier grass substrate

Napier grass (*Pennisetum purpureum* Scumach.) obtained was 2.5 - 3 months old after planting. After harvesting, it was immediately put into plastic and dried using shade drying machine at the Laboratory of Natural Product Isolation and Analysis, School of Life Science and Technology.

2.4. Napier grass sample preparation

Napier grass (*Pennisetum purpureum* Scumach.) that has been aged for 3 months was sun-dried for one day to reduce its water content from 89% to around 20-30% [23]. Napier grass was then oven roasted for 16 hours at 105 ºC, then cut into pieces and mashed with a blender (the size is between 10 and 35 mesh, about 2 mm) [9]. The material was then weighed every 15 minutes until it reached a constant mass.

2.5. Lignocellulose component analysis by the Chesson-Datta method

Lignocellulose fractionation from Napier grass was carried out using the Chesson-Datta method [10]. A total of 1 gram of dry sample (1 gram is the value (a) on the Chesson-Datta fraction calculation) was refluxed in 150 ml of distilled water at 100 ºC for two hours. The reflux solution was filtered with a Buchner funnel and the residue was dried in an oven at 150 ºC to a constant weight (weighed every 30 minutes). Dried residues which mass has stabled were weighed and a value of (b) was obtained.

The dry residue was then refluxed again using 150 ml of 0.5 M H_2SO_4 at 100 ºC for 2 hours. This reflux solution was rinsed with hot distilled water to neutralize the pH, and then filtered with Buchner, while the residue was dried in the oven to 105ºC until the residual mass was constant (weighed every 30 minutes). The constant residual mass is the value (c).

https://doi.org/10.5614/3bio.2020.2.1.5

The residue was then immersed in 10 ml of 72% v/v H_2SO_4 at room temperature for four hours and diluted to become 0.5 M H_2SO_4 solution, and then refluxed again for 2 hours at 100 ºC. The sample solution was rinsed with hot water and filtered with Buchner, and then dried in the oven for 2 hours or until the mass of residue was constant at 105 ºC. The constant residual mass is the value (d). The residue was then purified at 575 ºC. After obtaining the values a, b, c, d, and e, we then calculated the presence of hemicellulose, cellulose and lignin using the formulae as follows:

$$\text{Hot } Water\ Soluble\ Content\ (\%wt) = \frac{a-b}{a} x\ 100\% \quad (2.1)$$

$$Hemicellulose(\%wt) = \frac{b-c}{a} x\ 100\% \quad (2.2)$$

$$Cellulose\ (\%wt) = \frac{c-d}{a} x\ 100\% \quad (2.3)$$

$$Lignin\ (\%wt) = \frac{d-e}{a} x\ 100\% \quad (2.4)$$

$$Ash\ (\%wt) = \frac{e}{a} x\ 100\% \quad (2.5)$$

With :

- a : Initial sample dry weight (grams)
- b : Residual dry weight after refluxed in distilled water (grams)
- c : Residual dry weight after refluxed in 0.5 M sulfuric acid (grams)
- d : Residual dry weight after treatment in 72% sulfuric acid, dilution to a concentration of 0.5 M, and then refluxed in 0.5 M sulfuric acid (grams)
- e : Ash weight after it treated in the furnace (gram)

2.6. Developing *Phanerochaete chrysosporium* Viability Curves

This Viability Curve was made using the spread plate method [11]. Petri dishes containing PDAs were prepared under 27 sterile conditions. First, we made pure cultures from *P. chrysosporium* as many as 6 test tubes and cultured at the same conditions and times. The spore suspension was then harvested on a daily basis starting from day 2 of the PDA with the addition of 5 mL of harvest solution (0.85% NaCl w/v + Tween80 0.1% v/v) into the test tube. The spores were then harvested by gently rubbing the Ooze stick onto the PDA. The suspension was then homogenized using a vortex and 0.1-0.3 mL of it was taken to count the spores using a hemocytometer. The calculated suspensions were then diluted to a concentration of 10^3, 10^2, and 10^1 spores / mL (dilution with 0.9 mL suspension and 0.1 mL of harvest solution). After being diluted, 0.1 mL of each suspension was then taken to be put into a petri dish and spread evenly throughout the PDA. The same thing was done for days 3, 4, 5, and 6 with 2 repetitions for each day and concentration.

2.7. Biological Pretreatment

The first biological pretreatment used two species of fungi, namely soft-rot fungi (*Aspergillus niger*) and white-rot fungi (*Phanerochaete chrysosporium*), to degrade the lignin content in Napier grass. To carry out this pretreatment, it is first necessary to prepare a sample of Napier grass which is aged approximately 2-3 months and the propagated fungal spore as pretreatment materials.

Soft-rot fungi (*Aspergillus niger*) and white-rot fungi (*Phanerochaete chrysosporium*) cultures which have been inoculated on PDA medium in test tubes were then harvested with harvesting solution. The harvesting solution was made by mixing 0.85 grams of NaCl dissolved in 100 mL of distilled water (physiological solution of 0.85% NaCl). Then 0.1 mL of Tween80 (surfactant) was added and stirred until the solution became homogeneous. The homogenous solution was then put into the Erlenmeyer and covered with aluminum foil. The harvest solution was sterilized in an autoclave at 121 ºC (1.5 atm) for 15 minutes. *Aspergillus niger* would be harvested at its maximum growth time, which is four days when grown on the tilted PDA medium [12]. Likewise, *Phanerochaete chrysosporium* would be harvested in 5 days according to its maximum growth time.

Harvested fungal spores (5 mL of solution) were then counted by the number of fungal spore cells using a hemocytometer. Fungal spore concentrations were calculated and adjusted according to variations in fungal concentrations in **Table 1**.

Pretreatment time was determined on the basis of Ayed et al's (2013) study, which states that the greatest acquisition of LiP (Lignin Peroxydase) enzymes is in the pretreatment for 72 hours or 3 days, and according to Valencia and Meitiniarti (2017), in which the ability of *Aspergillus niger* biodelignification will continue to increase until the 7th day, which shows that research is needed for the 9th day as well [13] [15]. Furthermore, Napier grass which had been cut into pieces with a size of about 2 mm was sterilized by pasteurizing the 70 ºC (Low Heat Temperature) method for 3 times on manual heating, each for 15 minutes. Then, as much as 4 grams of Napier grass was put into a Rox bottle and additional nutrient solution was added until the Napier grass had an MC of 80% or with a nutritional ratio of 3.5 gram : 1 mL [16]. The Rox bottle containing Napier grass was inoculated with a ratio of 2 mL inoculum to 10 grams of Napier grass [17]. Additional nutrients given to Napier grass are listed in **Table 2**.

The Rox bottle was then put into the oven at 35 ºC and left for the treatment time [13]. During the treatment, the substrate was dried before it was analyzed for lignocellulose content using the Chesson data method. The pretreated grass was then rinsed with 1:50 of water between the acetate buffer (0.2 M, pH 4.5) and the grass then stirred at 130 rpm for 30 minutes to dissolve the reducing sugar found in the grass by

https://doi.org/10.5614/3bio.2020.2.1.5

soft-rot fungi (*Aspergillus niger*) [18]. Each pretreatment was done 2 times to get the best results with the smallest margin of error

2.8. Chemical Pretreatment

The second pretreatment given was using alkali ($Ca(OH)_2$). To carry out the chemical pretreatment, it is first necessary to prepare a sample of Napier grass aged approximately 2-3 months and also pretreatment materials that have been dissolved in accordance with certain variations in concentration. The variations used in this experiment are shown in **Table 3**.

Table 1 Time variation for pretreatment in Napier grass [13][14]

Type Treatments	Total Cell (cell/ g substrat)	Time (day)
JA1		1
JA2	10^6	3
JA3	10^7	5
JA4	10^8	7
JA5		9
JP1	10^6	28

Information : JA = *Aspergillus niger* JP = *Phanerochaete chrysosporium*

Table 2 Additional nutrients in pretreatment with fungi on Napier grass [17]

Nutrient	Weight (gram/L)
Yeast Extract	5
$(NH_4)_2SO_4$	1
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4$	0.2

Table 3 Variation of Alkali ($Ca(OH)_2$) pretreatment in Napier grass [19]

Variation	Component $Ca(OH)_2$ g/g on the substrat	Time
B1	0.02	6,24, and 96 hour
B2	0.1	
B3	0.5	

Information : B = Alkali.

After the Napier grass powder pertains a constant mass, the powder was then given a treatment using $Ca(OH)_2$ with a variation ratio of the concentration of chemical solvents and Napier grass powder according to **Table 3**. According to Xu et. al, (2008), the ratio between pretreatment solution and Napier grass substrate is 1: 10. This means that for every 1 gram of Napier grass substrate, a 10 mL pretreatment solution is needed [19]. A mixture of grass and water will form slurry or mud-like form. The substrate used in this study was 4 grams, so the required pretreatment solution was 40 mL. Repetition for each treatment was 3 times in order to obtain optimal results.

After the pretreatment was carried out in accordance with the time listed in **Table 3**, the solution and substrate of Napier grass were separated using a filter paper. The filtrate was then collected and continued with the reducing sugar test. The pretreated Napier grass substrate was then washed using 400 mL of distilled water to remove excess lime, reducing sugars, and by-products that could inhibit the hydrolysis process using enzymes [19]. After that the Napier grass was dried using an oven. After the Napier grass

https://doi.org/10.5614/3bio.2020.2.1.5

reached a constant weight, lignin, hemicellulose, and cellulose levels were analyzed using the Chesson-Datta method.

2.9. Sugar Reduction Analysis

Reducing sugar was analyzed using the DNS (dinitrosalycilic acid) method. Reducing sugar was measured on a spectrophotometer at a wavelength of 575 nm [20]. Reduction sugar analysis was carried out before and after the pretreatment to calculate the reduction of sugar contained in the treatment mixture.

The method for analyzing reducing sugars is as follows. First, 3 mL of the sample solution was put into a test tube and mixed with 3 mL of reagent DNS. Then the solution was closed and heated at a temperature of 90 ºC for approximately 5-15 minutes. The heated solution was added with 1 mL of Rochelle salt with a concentration of 40%. The solution was immediately cooled to room temperature and absorbance measured using a spectrophotometer at a wavelength of 575 nm.

3. Results and Discussion

3.1. The kinetics of the growth of the Phanerochaete chrysosporium

In this study, the kinetics of fungal growth was analyzed using *Phanerochaete chrysosporium* as a biological agent in biological pretreatment. There are many methods that can be used in experiments on the growth kinetics of an organism, such as the gravimetric, the counting chamber, the spectrophotometric method, and so on. The method used in this study is the Total Plate Count (TPC) method. TPC method is the most widely used method in this kind of analysis, because the colony can be seen directly with the eye without using a microscope. Another advantage of the spread plate method is that the grown fungi / bacteria can be spread evenly on the surface of the agar and this facilitates the calculation of the number of mold spores [21].

Based on data processed using the spread plate method, a fungi growth curve can be made by creating a viability curve between percent viability (%) of time and growth curve using a hemocytometer between cell counts / mL x 10^4 against time. Through these two curves, the time at which maximum growth occurs can be calculated. The two curves can be seen in **Figure 1** and **Figure 2**.

In **Figure 1**, it can be seen that the viability curve increases on days 2 and 3, while decreasing on day 4. This can be caused by the condition whereby the *Phanerochaete chrysosporium* culture on day 4 has a different treatment from the condition of the fungi culture on day 2, 3, 5, and 6. Differences in these conditions include initial culture conditions, PDA medium, and temperature of TPC testing which can cause a decreased growth. On the 5th day, the percentage of viability was obtained at the maximum value of 93.3%. This is supported by the growth curve in **Figure 2**, which produces the best growth on day 5.

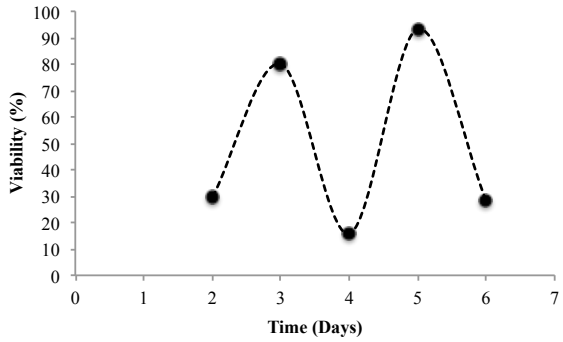


Figure 1 Viability (%) *P. chrysosporium*

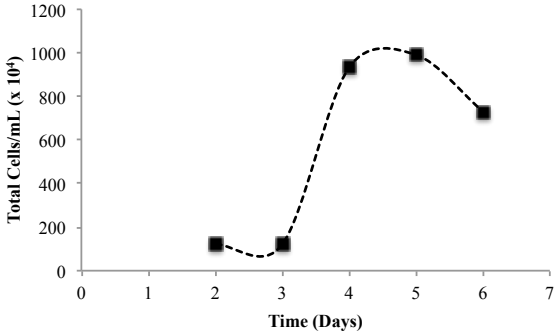


Figure 2 Growth Curve of *P. chrysosporium*

Furthermore, on the growth curve, it can be seen that on days 2 and 3, the growth of the *Phanerochaete chrysosporium* is still in the lag phase. This is in accordance with the study done by Yao and Nokes (2014) which states that in days 2-3, *Phanerochaete chrysosporium* is still in its lag phase of growth [22]. In the lag phase, the *Phanerochaete chrysosporium* is still adapting to its environment. It was further explained that the fungi enter an exponential phase on days 4-5. Here, a phase of slowing growth occurs.

In the deceleration phase, the fungi begin to run out of nutrients which causes the growth of biomass begins to decrease. In this study, the maximum growth was obtained on the 5th day, but it was different from the literature wherein the fungi continue to experience growth until the 11th day. This could be due to several factors such as the source of nutrition, temperature, and the strain of fungi used [23]. In our study, the medium used is slanted agar containing PDA medium. On the 5th to the 6th day, the fungi enter the death phase, where there is recorded decrease in cell count.

https://doi.org/10.5614/3bio.2020.2.1.5

From the two curves, the same results showed that the maximum growth of *Phanerochaete chrysosporium* was on the 5th day after inoculation. Fungal spore growth on PDAs was carried out at 37 ºC because it is the optimal temperature for the growth phase of *Phanerochaete chrysosporium* mycelium [24].

3.2. Effect of Variations in Alkali pretreatment on Lignocellulose Levels

The pretreatment process aims to break the lignin bond (delignification), in removing the lignin and some of the hemicellulose contents, damaging the crystalline structure of cellulose and increasing the porosity of the material [25]. The principle of lime ($Ca(OH)_2$) pretreatment is to degrade amorphous substances (such as lignin and hemicellulose) which makes the crystallinity increases. Lime ($Ca(OH)_2$) increases lignin degradation which makes enzymes work effectively by eliminating non-productive parts of the adoption and also increasing access to cellulose and hemicellulose [26]. Napier grass consists (wt%) of three main ingredients, namely hemicellulose at 37.5% cellulose, 26.5% hemicellulose and 14.9% lignin depending on the type and component of nutrients obtained [27].

From this study, measurements of lignocellulose component levels were carried out in the control and also in three variations of the concentration of lime pretreatment. All pretreatments in this study were carried out at the laboratory room temperature at Jatinangor, which ranged from 23 to 25 ºC and humidity ranging between 61 and 63%. The lignocellulose fraction in Napier grass can be seen in **Table 4**.

Table 4 Lignocellulose fraction of Napier grass (*Pennisetum purpureum* Scumach.)

Component	Content (%)
HWS	27.96
Hemicellulose	20.01
Cellulose	33.10
Lignin	17.82
Ash	1.12

The measurement of lignocellulose component levels was carried out using the Chesson-Datta method. The following is a display of lignocellulosic data resulting from pretreatment with a variation of lime concentration in **Figure 3**.

According to Mosier et al. (2005), pretreatment with lime ($Ca(OH)_2$) can impact the chemical composition of lignocellulose and change the chemical / physical structure of lignocellulose [6]. Lime pretreatment is proven to have a huge impact in increasing the area of cellulose that can be accessed by enzymes. In addition, lime pretreatment can also degrade lignin and destroy the hard lignin structure. However, lime pretreatment only affects slightly on the degradation of hemicellulose [6].

Delignification carried out by the lime pretreatment process in Napier grass samples in this experiment reduced some components of HWS, lignocellulose, cellulose, and lignin, as shown in **Figure 3**. The best delignification conditions can be achieved if the results show low lignin values and high cellulose values, or a low ratio of lignin to cellulose. The factors which also affect the value of lignin and cellulose are lignin degradation and the recovery of solids and cellulose. This needs to be taken into account [28].

The top priority of the lignocellulose component is lignin. Alkali pretreatment can cause swelling of biomass, which can make enzymes or other biocatalyst agents to access biomass more easily [29]. Alkali pretreatment allows breaking of the lignin bonds and changing the crystalline structure of cellulose.

In **Figure 3**, it can be clearly seen that the control sample has a high lignin level of 17.82 wt% and cellulose content of 38%. All lime pretreatments with concentrations of 0.5, 0.1 and 0.02 on average reduce the levels of lignin and HWS. This is due to the nature of lime that can degrade lignin by destroying its structure within a relatively high pH with concentrations of 0.5 g/g, 0.1 g/g, and 0.02 g/g are 12.3 wt%, 12.8 wt%, and 13.2 wt%, respectively [30]. This is also true to the hemicellulose content obtained in this study, where the result only differs slightly from the control. This is in accordance with Mosier, et al. (2005) who asserts that the impact of lime pretreatment on hemicellulose only slightly degrades hemicellulose [6].

In **Figure 3**, it is also seen that the smallest lignin content is found in lime pretreatment with a concentration of 0.5 g / g substrate in 96-hour time with 7.4% lignin level. However, the level of cellulose obtained was only 25% and relatively small when compared with controls. The largest cellulose content was found in lime pretreatment and proves to produce the largest lignin reduction of 58.47%.

It will be difficult to determine pretreatment with maximum concentration and time when viewed from each aspect of lignocellulose. This is because the results are tightly close and hard to compare. As an alternative, the difference can be seen by reviewing the aspect of lignin to cellulose ratio, considering that the aim is to obtain as lowest lignin value and highest cellulose value as possible. **Figure 4** shows the ratio between lignin and cellulose in each treatment.

Figure 4 shows that the concentration of lime pretreatment with the highest cellulose content and lowest lignin content was 0.1 gram / gram of Napier grass in 24 hours, with a ratio of 24.56%. These results differ only slightly with the concentration of 0.02 gram / gram of Napier grass in 96 hours, as well as with a concentration of 0.5 gram

https://doi.org/10.5614/3bio.2020.2.1.5

/ gram of Napier grass in 6 hours, with ratios of 24.67% and 26.79%, respectively. This is also supported by the results of the reducing sugar contained in the pretreatment hydrolyzate in **Figure 5**.

The results shown in **Figure 5** also demonstrate that the concentration of lime pretreatment with the highest reducing sugar content is 0.1 gram / gram of Napier grass within 24 hours of treatment. These results were obtained in accordance with the results of lignin degradation with lime on switchgrass, with the best concentration of 0.10 g/gram of biomass for 24 hours at 50 ºC [19].

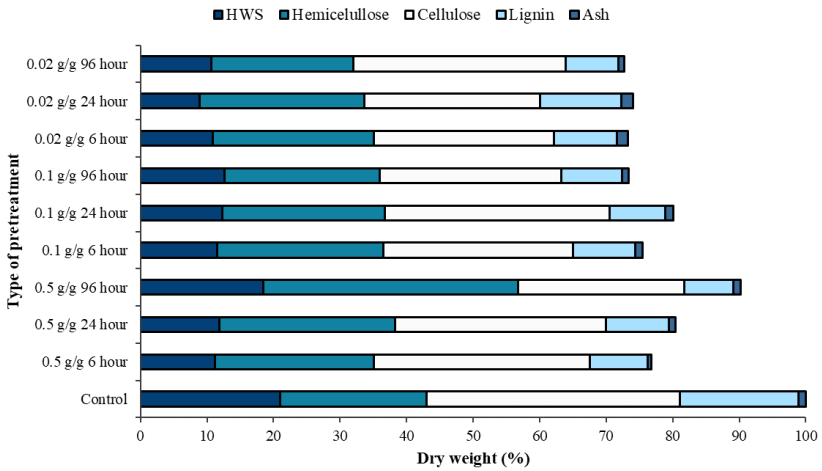


Figure 3 Lignocellulose fraction in lime ($Ca(OH)_2$) pretreatment

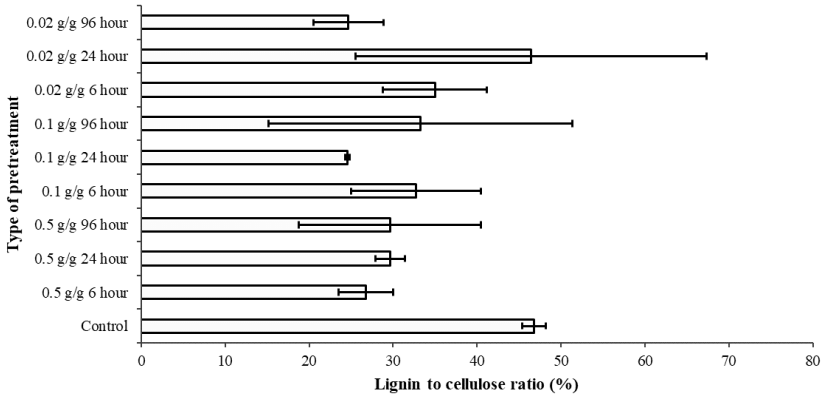


Figure 4 Lignin to Cellulose Ratio on the lime pretreatment result'

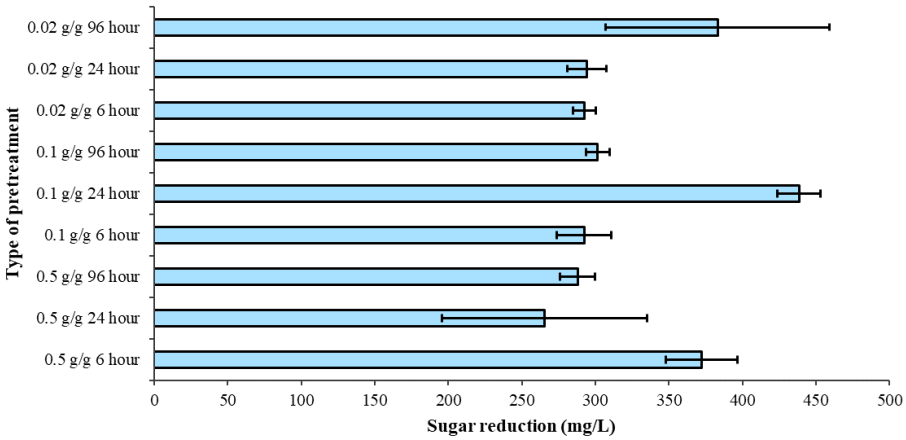


Figure 5 Reducing Sugar Level (mg/L) on hydrolyzate from the lime pretreatment

https://doi.org/10.5614/3bio.2020.2.1.5

3.3. Effect of variations in concentration and time of biological pretreatment with Aspergillus niger on levels of lignocellulose

Biological pretreatment is a preliminary treatment of lignocellulosic biomass to break the hard and crystal-shaped lignin bonds. The process uses biological agents (fungi) that possess enzymes as weapons to break down lignin [31]. Biological pretreatment is an efficient, environmentally friendly, and inexpensive pretreatment [32]. Microorganisms such as brown- and white-rot fungi are commonly used to degrade lignin and hemicellulose in waste biomass such as agricultural waste [33]. On the other hand, soft-rot fungi, which are fungi of the ascomycetes class, has a very strong property of degrading carbohydrates in wood, and some of these fungi can show a significant ability in breaking down the structure of lignin [34]. However, lignin degradation by soft-rot fungi is still little studied.

In general, the enzymes produced by these fungi are one of the three main enzymes that degrade lignin, namely LiP, MnP, and Laccase [35]. **Figure 6** to **13** illustrates lignocellulosic component data ranging from HWS, hemicellulose, cellulose, lignin and ash resulting from pretreatment with *Aspergillus niger* with varying fungal concentrations and pretreatment times.

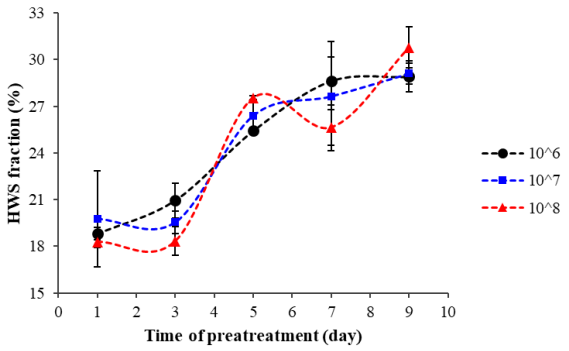


Figure 6 HWS Fraction at each fungi concentration

Data on the treatment of *Aspergillus niger* in HWS fraction was analysed using the Duncan's Multiple Range Test ($p < 0.05$), and showed a significant increase between day 1 and day 9. This could be due to *Aspergillus niger* proven to be able to produce cellulose- and hemicellulose-breaking enzymes, one of which is xylanase [36] [37]. The enzyme can break down cellulose and hemicellulose into simple sugars that can be dissolved in the HWS fraction, thus causing the value of the HWS fraction to increase. HWS consists of several simple components that dissolve easily in water, such as some simple carbohydrates, proteins, and inorganic compounds [4].

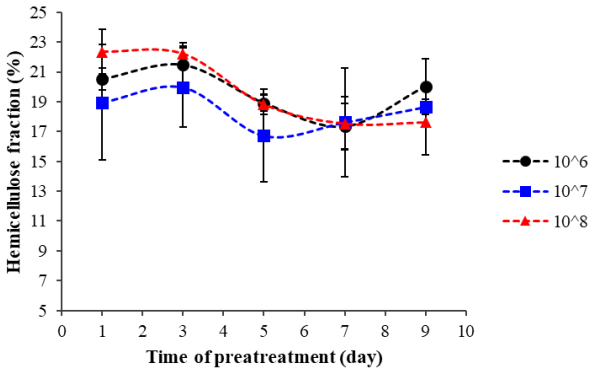


Figure 7 Hemicellulose fraction at each fungi concentration

When viewed from the hemicellulose fraction, the results show that there was a significant decrease between day 1 and day 9. This can be explained due to the ability of the *Aspergillus niger* to break down hemicellulose into simple sugars (pentose (xylose and arabinose), hexose (manose, glucose, and galactose), and sugar acids [38], which will be used as a source of C in its growth [39].

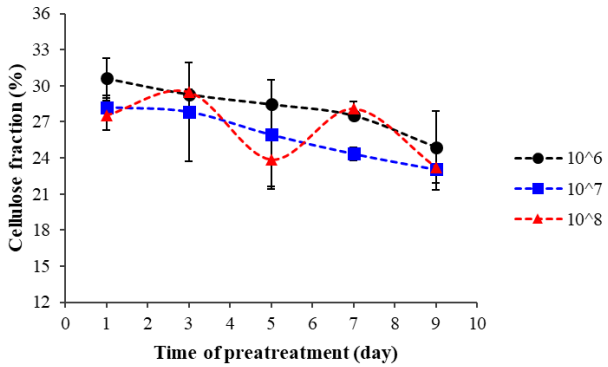


Figure 8 Cellulose fraction at each fungi concentration

The same holds true with the cellulose fraction, where a significant decrease was recorded on day 1 and day 9. This is also due to the *Aspergillus niger* having the ability to convert the cellulose fraction into simple sugars (D-glucose) using cellulase enzymes such as xylanase [36]. This is what causes cellulose fraction content to decrease from day 1 to day 9. In the pretreatment process, the cellulose fraction is expected not to experience a reduction because if cellulose is reduced in the pretreatment process, the saccharification process in the fermentation will not run optimally [25].

Data on lignin fraction that have been analysed using Duncan's Multiple Range Test ($p < 0.05$) showed that a significant decrease from day 1 to day 9 occurred. Lignin fraction itself is the main parameter in the (chemical and biological) pretreatment process. Some journal articles state that lignin levels have a considerable influence on the acquisition of ethanol from pretreated biomass [40].

DOI: 10.5614.3bio.2020.2.2.2

Although lignin is the main parameter, many other factors such as lignin composition, chemical structure of lignin, as well as complex bonds between lignin and carbohydrates in biomass can have an important impact on biomass digestibility. Although lignin is the hardest component in biomass, it can be concluded that reducing lignin levels can improve the ability to access substrate in bioethanol production [41].

It should be noted that the main purpose of pretreatment is to obtain a low ratio of lignin to cellulose. **Figure 10** illustrates the ratio between lignin and cellulose in each fungal treatment.

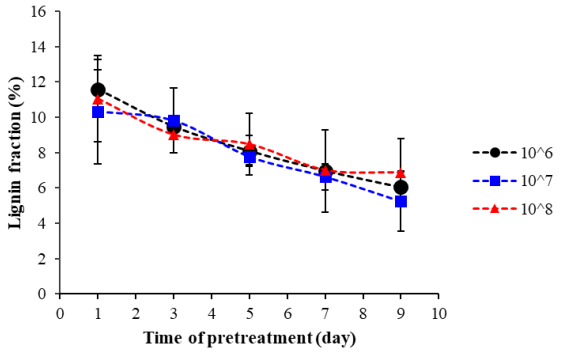


Figure 9 Lignin fraction at each fungi concentration

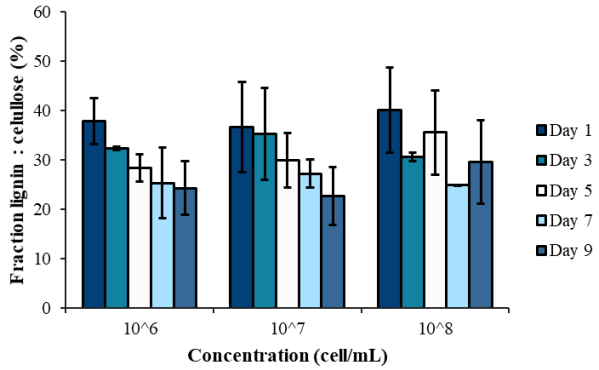


Figure 10 Lignin : Cellulose ratio in pretreatment with *Aspergillus niger*

By analyzing the data using Duncan's Multiple Range Test ($p < 0.05$) on the ratio of lignin to cellulose, it was shown that the smallest value was found on the 9th day at a concentration of 10^7. In this test, the smaller the value of the ratio of lignin to cellulose, the smaller the lignin level is compared to the cellulose fraction, and the more effective a pretreatment is. This means that the best pretreatment using *Aspergillus niger* in this study was 9 days pretreatment using 10^7 concentrations. This is supported by observations of *Aspergillus niger* growth at 10^7 concentrations that are more evenly distributed throughout the surface of Napier grass. At concentrations of 10^6 and 10^8, fungi growth was concentrated only at a few points of Napier grass. **Figure 11** documents the results of the pretreatment with the *Aspergillus niger* on day 9.

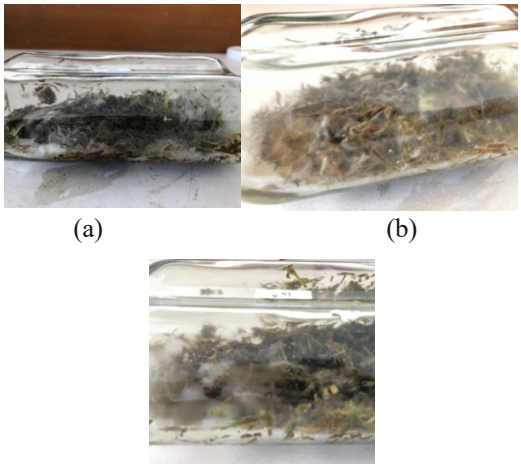


Figure 11 Pretreatment with *Aspergillus niger* spore at concentrations (a)10^6, (b)10^7, (c)10^8 cells/mL and day 9

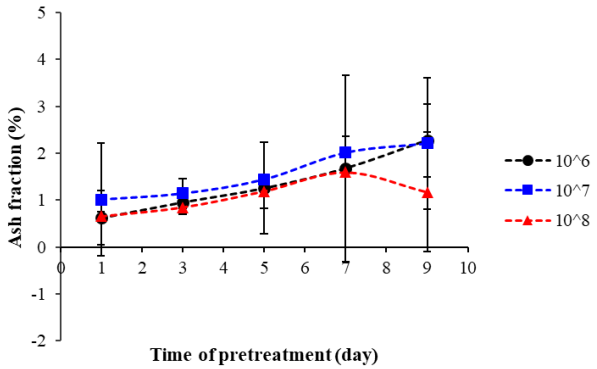


Figure 12 Ash fraction at each fungi concentration

The results of data analysis using Duncan's Multiple Range Test ($p < 0.05$) in the ash fraction showed a significant increase on day 1 and day 9. The ash fraction showed the presence of inorganic compounds in Napier grass cells. Inorganic compounds such as minerals (calcium, magnesium, phosphorus, chlorine, sodium, and sulfur) are present in several parts of the cell wall [42]. If the cell wall component is broken down / extracted, the inorganic compounds such as minerals will come out. Therefore, in this study the ash fraction content tends to increase due to the *Aspergillus niger* which damages cell walls and makes mineral levels increase from day 1 to day 9.

Reducing sugar is a sugar (carbohydrate) class that has the ability to reduce electron-accepting compounds [43]. Examples of sugars that include reducing sugars are glucose, mannose, fructose, lactose, maltose, and others. Generally, the reducing sugars produced are closely related to enzyme

https://doi.org/10.5614/3bio.2020.2.1.5

activity, where the higher the enzyme activity, the higher the reducing sugars produced [44]. With the results of data analysis using the Duncan's Multiple Range Test ($p < 0.05$) on the reducing sugar fraction, we can see that there is an increase in reducing sugar levels from day 1 and day 9. This is because the *Aspergillus niger* can produce enzymes to break down the cellulose and hemicellulose components, one of which is xylanase into simple sugars [36] [37].

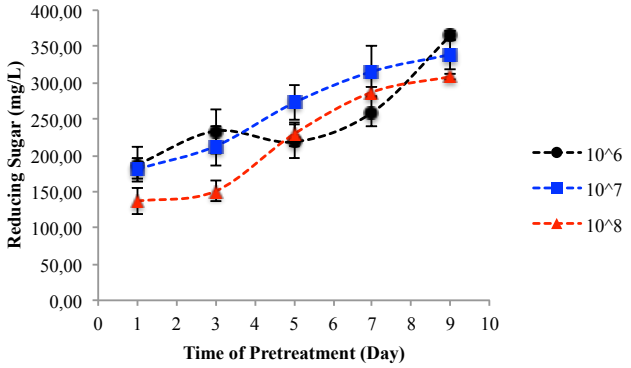


Figure 13 Reducing Sugar Levels at each fungi concentration

3.4. *Biological Pretreatment Comparison between Phanerochaete chrysosporium and Aspergillus niger*

This study uses two species of fungi from different classes, namely white rot fungi and soft rot fungi. The *Phanerochaete chrysosporium* has become a model of organism from which we can learn the extent to which fungi are able to degrade lignin [45]. In this sense, *Phanerochaete chrysosporium* is used in this study as a comparison to the main biological agent used, which is *Aspergillus niger*. This study seeks to prove that *Aspergillus niger* has the potential to be used as a biodelignification agent.

In this study, the treatment of *Phanerochaete chrysosporium* was carried out under the same conditions as that of *Aspergillus niger* and used at optimal concentration, temperature, and moisture content [14]. **Table 5** shows a comparison of lignocellulosic components along with reducing sugars between *Aspergillus niger* and *Phanerochaete chrysosporium* under the same conditions at 35 °C, with concentrations of 10^6 cells / mL and the same medium.

Table 5 Comparison of lignocellulosic component and reducing sugar between *Aspergillus niger* and *Phanerochaete chrysosporium*

Component	*Aspergillus niger*	*Phanerochaete chrysosporium*
Time of pretreatment (day)	9	28 [14]
HWS (%)	28.904 ± 0.997	28.081 ±0.899
Hemicellulose (%)	20.022 ± 1.867	12.150 ± 0.631
Cellulose(%)	24.880 ± 2.991	10.380 ± 0.976
Lignin (%)	6.044 ± 0.905	3.181 ± 0.222
Ash (%)	2.275 ± 0.778	0.407 ± 0.331
Reducing sugar (mg/L)	365.43 ± 5.534	325.45 ± 7.566

When viewed from hemicellulose and cellulose content, pretreatment using *Aspergillus niger* is superior to *Phanerochaete chrysosporium* because the former relies on a wide spectrum of sugar sources. Likewise, the reducing sugar produced by the *Aspergillus niger* is superior to that of *Phanerochaete chrysosporium*. This is because the optimal pretreatment time of the *Phanerochaete chrysosporium* is longer than the *Aspergillus niger,* which causes greater cellulose consumption compared to the latter. The rate of sugar consumption possessed by both fungi is relatively the same [46].

As for the lignin parameter, when the level of lignin is compared with the amount of cellulose, *Aspergillus niger* is superior to *Phanerochaete chrysosporium*, with the percentage of lignin to cellulose to be 24.3% and 30.645%, respectively. This value indicates that *Aspergillus niger* is able to process more cellulose as raw material for producing ethanol in the saccharification and fermentation process.

3.5. *Comparison between Biological and Alkali Pretreatment*

In this study, two types of pretreatment are compared on the basis of optimal time and concentration. The parameters used to compare the effectiveness of pretreatment are lignocellulosic components which consist of HWS,

https://doi.org/10.5614/3bio.2020.2.1.5

Hemicellulose, Cellulose, Lignin, Ash, and Reducing Sugar fractions. Comparison between Biological pretreatment using the *Aspergillus niger* and Alkali pretreatment using lime ($Ca(OH)_2$) is shown in Table 6.

When viewed from the components of hemicellulose, cellulose, and reducing sugars, Alkali pretreatment is superior to Biological pretreatment. This is because Alkali pretreatment can strongly affect lignin degradation but only slightly degrades hemicellulose, cellulose, and other sugars [6]. As explained by Liong et al. (2012), biological pretreatment is definitely lower than alkali pretreatment when it comes to glucose acquisition [14]. In short, the reason is that fungi need a longer time to consume lignin.

However, because the main parameter of this study was the ratio of lignin to cellulose, the biological pretreatment using *Aspergillus niger* was superior compared to the Alkali pretreatment, with a ratio of 22.7% and 24.5%, respectively. This can be caused by three things. First, the enzyme produced by *Aspergillus niger* will achieve maximum activity on day 7, therefore on days 7 to 9, *Aspergillus niger* is in maximum condition to produce cellulase, hemicellulase, or delignification enzymes [47]. The second reason is Alkali's ability to degrade lignin. In the reaction between alkali and lignocellulose, the main goal is degradation of lignin, but bases can also degrade cellulose and hemicellulose. This can reduce the ability of alkalis to degrade lignin [48]. Furthermore, because the lignin fraction has a reactive group, the condensation reaction between lignin components can inhibit the ability of alkali in delignification. Lastly, in the alkali pretreatment of lignin, conjugated acids can form. When acid is formed, it can cause the consumption of alkaline components and lower the pH value [48].

pH testing was also carried out on lime ($Ca(OH)_2$) pretreatment to determine the formation of conjugated acid. The pH at the optimal alkali pretreatment conditions with lime ($Ca(OH)_2$) in a concentration of 0.1 gram / gram substrate for 24 hours was 7.397 ± 1.060. Meanwhile, in the initial pretreatment conditions, the pH given was around 12.8. It can therefore be concluded that the formation of conjugated acid causes pH to decrease by 5.403. The lime ($Ca(OH)_2$) pretreatment does not run optimally under these conditions.

4. Conclusions

From our study, we can see that the most optimal biological pretreatment time using *Aspergillus niger* is 9 days with the most optimal fungal spore concentration to degrade lignin is 107 cells/mL. Comparison between pretreatment with *Aspergillus niger* and *Phanerochaete chrysosporium* towards better delignification ability in optimal conditions shows that the former could give better results, using hemicellulose, cellulose and reducing sugar concentration parameters. In alkali pretreatment using lime (Ca(OH)2), the results show that the most optimal time and concentration of pretreatment in degraded lignin is 0.1 gram / gram substrate with a pretreatment time of 24 hours (1 day). Comparison between Alkali pretreatment using (Ca(OH)2) and Biological pretreatment using *Aspergillus niger* show that the latter was better, seen using the main parameter of ratio between lignin and cellulose content.

References

[1] Nugroho, R. (2014). Kebijakan Publik di Negara-Negara Berkembang.

[2] Syam, K. L., Farikha, J., & Fitriana, D. N. (2009). Pemanfaatan Limbah Pod Kakao Untuk Menghasilkan Bioetanol Sebagai Sumber Energi Terbarukan.

[3] Negawo, A. T., Teshome, A., Kumar, A., Hanson, J., & Jones, C. S. (2017). Opportunities for Elephant (Pennisetum purpureum) Improvement Using Molecular Genetics. Agronomy , 7 (28).

[4] Isroi. (2008). Peningkatan Digestibilitas dan Perubahan Struktur Tandan Kosong Kelapa Sawit oleh Pretreatment Pleurotus floridanus dan Asam Fosfat. Disertasi, Universitas Gadjah Mada, Sekolah Pascasarjana – Program Studi Bioteknologi, Yogyakarta.

[5] Sewalt, V. J., Glasser, W. G., & Beauchemin, K. A. (1997). Lignin impact on fiber degradation .3. Reversal of inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. J Agric Food Chem , 5 (45), 1823-1828.

[6] Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., et al. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology (96), 673-686.

[7] Khrishna, H. S., & Chowdary, G. V. (2000). Optimization of simultaneous saccharification and fermentation for the production of ethanol from lignocellulosic biomass. J Agric Food Chem , 5 (48), 1971-1976.

[8] Chen, S., Zhang, X., Singh, D., Yu, H., & Yang, X. (2010). Biological pretreatment of lignocellulosics: potential, progress and challenges. Biofuels , 1 (1), 177-199.

[9] Rahnama, N., Mamat, S., Shah, U. K., Ling, F. H., Rahman, N. A., & Ariff, A. B. (2013). Effect of Alkali Pretreatment of Rice Straw on Cellulase and Xylanase Production by Local Trichoderma harzianum SNRS3 under Solit State Fermentation. 8 (2), 2881-2896.

[10] Kienzle, R. (1981). Acidogenic Fermentation of Lignocellulose-Acid Yield and Conversion of Components. Biotechnology and Bioengineering, , 23, 2167-2170.

[11] Fugelsan, K. C., & Edwards, C. G. (2007). Wine Microbiology : Practical Applications and Procedures. 2, 226-236.

[12] Zuliansah, W., & Abduh, M. Y. (2018). Pengaruh Lama Waktu Kultivasi Aspergillus niger dengan Metode Solid State Fermentation (SSF) Terhadap Kandungan Total Flavonoid dari Daun Lemon (Citrus limon. L). Institut Teknologi Bandung, Sekolah Ilmu dan Teknologi Hayati. Bandung: Repository Tugas Akhir SITH-ITB.

https://doi.org/10.5614/3bio.2020.2.1.5

[13] Ayed, L., Chammam, N., Asses, N., & Hamdi, M. (2013). Optimization of Biological Pretreatment of Green Table Olive Processing Wastewaters Using Aspergillus niger. J. Bioremed Biodeg , 5, 212.
[14] Liong, Y. Y., Halis, R., Lai, O. M., & Mohamed, R. (2012). Conversion of Lignocellulosic Biomass from Grass to Bioethanol Using Materials Pretreated with Alkali and White Rot Fungi, Phanerochaete chrysosporium. BioResources , 7 (4), 5500-5513.
[15] Valencia, P. E., & Meitiniarti, V. I. (2017). Isolasi dan karakterisasi jamur ligninolitik serta perbandingan kemampuannya dalam biodelignifikasi. Scripta Biologica , 4 (3), 171-175.
[16] Correa, M. G., Portal, L., Moreno, P., & Tengerdy, R. P. (1999). Mixed culture solid substrate fermentation of Trichoderma reesei with Aspergillus niger on sugar cane bagasse. Bioresource Technology , 173-178.
[17] Pensupa, N., Jin, M., Kokolski, M., Archer, D. B., & Du, C. (2013). A solid state fungal fermentation-based strategy for the hydrolysis of wheat straw. Bioresource Technology .
[18] Alessandra, B., Farias, F. C., Motter, F. A., Paula, D. H., Richard, P., Krieger, N., et al. (2014). Pectinase Activity Determination: An Early Deceleration in the Release of Reducing Sugars Throws a Spanner in the Works! 9 (10).
[19] Xu, J., Cheng, J. J., Sharma-Shivappa, R. R., & Burns, J. C. (2008). Lime Pretreatment of Switchgrass for Bioethanol Production. ASABE Annual International Meeting. ASABE Rhode Island Convention Center.
[20] Miller, G. L. (1959). Use of dinitro salicylic acid reagen for reagen for determination of reducing sugar. Anal. Chem (31), 426-428.
[21] Budiyanto, M. K. (2011). Teknik Membuat Biakan Murni. Kajian Mikrobiologi Umum. Pondok Ilmu.
[22] Yao, W., & Nokes, S. E. (2014). Phanerochaete chrysosporium pretreatment of biomass to enhance solvent production in subsequent bacterial solid-substrate cultivation. Biomass and Bioenergy (62), 100-107.
[23] Isroi, Millati, R., Syamsiah, S., Niklasson, C., Cahyanto, M. N., Lundquist, K., et al. (2011). Biological Pretreatment of Lignocellulos es with White-Rot Fungi and Its Applications : A Review. BioResources , 6 (4), 5224-5259.
[24] Asther, M., Capdevila, C., & Corrieu, G. (1988). Control of lignin peroxidase production by Phanerochaete chrysosporium INA-12 by temperature shifting. Applied and Environmental Microbiology (54), 3194-3196.
[25] Alvira, P., Tomas-Pejo, E. M., Ballesteros, M., & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology (101), 4851-4861.
[26] Yang, B., & Wyman, C. E. (2007). Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels, Bioprod. Bioref , 2, 26-40.
[27] Yasuda, M., Takeo, K., Matsumoto, T., Shiragami, T., Sugamoto, K., Matshushita, Y.-i., et al. (2013). Effectiveness of Lignin-Removal in Simultaneous Saccharification and Fermentation for Ethanol Production from Napiergrass, Rice Straw, Silvergrass, and Bamboo with Different Lignin-Contents. Sustainable Degradation of Lignocellulosic Biomass - Techniques, Applications and Commercialization .
[28] Minmunin, J., Limpitipanich, P., & Promwungkwa, A. (2015). Delignification of Elephant Grass for Production of Cellulosic Intermediate. Energy Procedia (79), 220-225.
[29] Restiawaty, E., & Dewi, A. (2017). Comparison of Pretreatment Methods on Vetiver Leaves for Efficient Processes of Simultaneous Saccharification and Fermentation by Neurospora sp. IOP Journal of Physics: Conference Series (877).
[30] Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial & Engineering Chemistry Research , 8 (48), 3713-3729.
[31] Maurya, D. P., Singla, A., & Negi, S. (2015). An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. 3 Biotech .
[32] Wan, C., & Li, Y. (2012). Fungal pretreatment of lignocellulosic biomass. Biotechnology advances , 30 (6), 1447-1457.
[33] Halis, R., Tan, H. R., Ashaari, Z., & Mohamed, R. (2012). Biomodification of Kenaf using White Rot Fungi. BioResources , 1 (7), 984-987.
[34] Blanchette, R. A. (1995). Degradation of the lignocellulose complex in wood. Canadian Journal of Botany , 1 (73), 999-1010.
[35] Hatakka, A. (2001). Biodegradation of lignin. 129-180.
[36] Mussatto, S. I., & Teixeira, J. (2010). Lignocellulose as raw material in fermentation processes. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology (Méndez-Vilas, A, Ed) , 2, 897-907.
[37] Gawande, P. V., & Kamat, M. Y. (1999). Production of Aspergillus xylanase by lignocellulosic waste fermentation and its application. Journal of Applied Microbiology (87), 511-519.
[38] Saha, B. C. (2003). Hemicellulose bioconversion. J. Ind. Microbiol. Biotechnol (30), 279-291.
[39] Sun, Y., & Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol , 1 (83), 1-11.
[40] Castillo, R. D., Parra, C., Troncoso, E., Franco, H., Pena, S., & Freer, J. (2014). Nir Spectroscopy applied to the Characterization and Selection of Pre-Treated Materials from Multiple Lignocellulosic Resources for Bioethanol Production. J. Chil. Chem. Soc. , 59 (1).
[41] Gutierrez, E. D., Amul, K. M., Carpio, R. M., & Toledo, A. R. (2015). Effect of Selected Fermentation Parameters on Bioethanol Production from Ripe Carabao Mango (Mangifera indica) Peelings. Asia Pacific Journal of Multidisciplinary Research , 3 (4).
[42] Mastuti, R. (2016). Nutrisi Mineral Tumbuhan. Modul 2-1 : Elemen esensial: Fungsi, absorbsi dari tanah oleh akar, mobilitas, dan defisiensi .
[43] Oktana, R. (2013). Gula Reduksi.
[44] Gaman, P. M., & Sherington, K. B. (1992). Ilmu Pangan: Pengantar Ilmu Pangan Nutrisi dan Mikrobiologi.
[45] Kirk, T. M., & Farrell, R. (1987). Enzymatic ‘‘combustion’’: the microbial degradation of lignin. Annu Rev Microbiol , 41, 465-505.

https://doi.org/10.5614/3bio.2020.2.1.5

[46] Reddy, M. K., Maruthi, Y. A., & Lakshmi, K. A. (2010). Aspergillus flavus and Phanerochaete chrysosporium as potential delignifying mycoflora of dump yard: a case study. Rasayna J. Chem. , 3, 438-444.
[47] Aliyu, N., Kassim, H., Aliyu, H., Abu, R. J., & Adamu, Z. (2017). Comparative Production of Cellulase from Chemical and Biological Pretreated Groundnut Husk. Advances in Research , 10 (6), 1-7.
[48] Ramirez, R. S. (2010). Kinetic Modeling and Assessment of Lime Pretreatment of Poplar Wood. Dissertation, Texas A&M University, Chemical Engineering.

https://doi.org/10.5614/3bio.2020.2.1.5

3Bio Journal of Biological Science, Technology and Management
Guidelines for Authors

Submitting your manuscript: Manuscript for publication should be submitted electronically to 3BIO: Journal of Biological Sciences, Technology and Management to facilitate rapid publication and minimize administrative costs. All manuscripts should be submitted through online submission system. A user ID and password for the site can be obtained on first use. Online submission ensures the quickest possible review and allows authors to track the progress of their papers. In order to submit a NEW Manuscript to 3BIO: Journal of Biological Sciences, Technology and Management, you must be a registered user of 3BIO Journal, if you do not register, please register before you submit a NEW Manuscript. Submissions by anyone other than one of the authors will not be accepted. The submitting author takes responsibility for the paper during submission and peer review.

Other relevant correspondence should be sent to The Editorial Office of the 3BIO Journal of Biological Science, Technology and Management, c/o Dr. Rudi Dungani, School of Life Sciences and Technology, Institut Teknologi Bandung, Jl. Ganesha 10 Bandung 40132, Indonesia. Phone +62-22-2500258 Fax +62-22-2534107 E-mail: support3BIO@sith.itb.ac.id

Type of articles: Articles may be in the form of Research article, Review article or Short communication. Research articles are reports of recent advances on the research of selected topics. Short communications are concise, but independent report representing a significant contribution in the field.

Manuscript form: All manuscript must be written in English. Please ensure the following when submitting your manuscript to 3BIO:

1. The manuscript has not been submitted/published earlier in any journal and is not under consideration for publication elsewhere (or an explanation has been provided in Comments to the Editor section).
2. All authors have seen and approved the manuscript and have contributed significantly for the manuscript.
3. The submitted file should be in OpenOffice, Microsoft World, RTF, or WordPerfect document file format.
4. Cover letter should be written in Comments to the Editor section or submitted as a separate file in the Supporting Files section.
5. The text is single-spaced, uses a 12-point font, employs italics rather than underlining (except with URL addresses), and all illustrations, figures and tables are place within the text at the appropriate points, rather than at the end of the document.
6. The text adheres to the stylistic and bibliographic requirements outlined in the Author Guidelines, which is found in the 'Guidelines to references' page.
7. Where available, URLs for the references should be provided.

Referencing: 3Bio uses Vancouver citation style. Vancouver is a numbered referencing style commonly used in medicine and science, and consists of:

- Citations to someone else's work in the text, indicated by the use of a number.
- A sequentially numbered reference list at the end of the document providing full details of the corresponding in-text reference.

It follows rules established by the International Committee of Medical Journal Editors, now maintained by the U.S. National Library of Medicine. It is also known as Uniform Requirements for Manuscripts submitted to Biomedical Journals.

File Size and Format: Manuscripts will be distributed to reviewers via the Web. However, reviewers who use telephone modems may experience unacceptable download delays if the files are too large. A number of simple tricks can be used to avoid unnecessarily large files. Do not scan pages of text. Do not scan printed Figures unless no original digital document exists.

If a scanned figure is unavoidable, please use Adobe PhotoShop or a similar program to edit the file and reduce the file size (not necessarily the image size) as much as possible before submission. For example, crop the picture to exclude surrounding "white space." Do not carelessly use colour. Black and white line drawings or gray-scale figures should not be saved as color documents; this will increase file sizes without increasing the information content of the file. Do not use colour unless absolutely needed to convey information.

Readability: A paper may be returned to the corresponding author for no other reason than that it suffers due to poor English. Papers must be understandable and communicate an unambiguous message. The editors and staff can make only a limited number of edits, and it is the responsibility of the authors to obtain help from a colleague who is fluent in English if that is needed. Most problems occur when there are nuances in meaning, and the authors bear the primary responsibility for clarity. Poor English may ultimately be a reason to refuse a paper.

3Bio JOURNAL OF BIOLOGICAL SCIENCE, TECHNOLOGY AND MANAGEMENT

Table of Content

School of Life Sciences and Technology
Institut Teknologi Bandung - Indonesia