Delignification and determination of sugar concentration in fertilizer as the preliminary process of bioethanol production by *Aspergillus fumigatus*

K Lois, B Iswanto and A Rinanti*

Department of Environmental Engineering, Faculty of Landscape Architecture and Environmental Technology, Universitas Trisakti, Jakarta, Indonesia

*astririnanti@trisakti.ac.id

Abstract. Water hyacinth is an aquatic weed that causes an imbalance of the ecosystem hence, it needs to be processed into useful products. This research conducted the degradation of lignocellulose-containing biomass, utilizing *Aspergillus fumigatus* fungi to form bioethanol. This study was initiated with the cultivation of *A. fumigatus* in Potato Dextrose Broth media and the preparation of water hyacinth as a substrate up to 25 mesh in size. Pretreatment was carried out using varying ratios of fungi to substrate of 1: 1, 1: 5, and 1:10 and contact time is 24, 72, and 120 hours. Furthermore, the lignin level was determined using the gravimetric method and hydrolysis was carried out afterwards in order to prepare the sugar for the fermentation procedure. The sugar content produced was analyzed using the DNS method. In this study, the highest allowance for lignin at the pretreatment stage was 25.9%, this produced the highest sugar at the hydrolysis stage (0.5 g/L). Fungi *A. fumigatus* functions as a biocatalyst which is suitable in the delignification process as a pretreatment and hydrolysis. A fermentation procedure and further research is needed to determine the potential of water hyacinth as a raw material in the production of bioethanol.

1. Introduction
An upsurge in the quantity of organic waste present in waterways causes excess nutrients (eutrophication) which further triggers the growth of bacteria and aquatic weeds, for example, water hyacinth (*Eichhornia crassipes*). These weeds absorb nitrogen and phosphorus compounds from contaminated water and they can also deplete nutrients and oxygen quickly from water bodies, which affect flora and fauna, causing imbalance in the aquatic ecosystems. In addition, its high evapotranspiration has lead to the incidence of water crisis in places where it grows, therefore, efforts are needed to turn water hyacinth into something useful [1]. Furthermore, it has been observed to be a raw material for lignocellulose which has the potential to produce second generation bioethanol.

Bioethanol is a biofuel that can serve as an environmentally friendly alternative fuel [2]. Structurally, water hyacinth has monosaccharide and polysaccharide unit, in general, the polymer contains cellulose, a sugar homopolymer and hemicellulose, formed from 5 to 6 carbon monomers. Furthermore, it also contains lignin, a complex polymer with high molecular weight that is tightly bound to carbohydrates [3]. Water hyacinth has cellulose content (19% - 25%), hemicellulose (15% - 40%), and lignin (3% - 15%) [4-7].
There are four main steps taken in the production of bioethanol, these include, pretreatment, hydrolysis, fermentation, and distillation [8]. Pretreatment or delignification is needed because lignin functions as a form of ‘glue’ that provides structural strength to biomass fibers. It acts as a wall for each solution or enzyme by connecting both hemicellulose and cellulose, thus preventing the penetration of lignocellulolytic enzymes into interior lignocellulose structures. The lignin bond must therefore be cut in order to facilitate the hydrolysis process [9].

This procedure is followed by hydrolysis, which is a bio-chemical process that degrades cellulose at the pretreatment stage into sugar that is further fermented with yeast [10]. Hydrolysis can be conducted using acid, base, or enzymes and enzymatic hydrolysis has widely been accepted because of its low energy requirements and environmental impact [11]. The conversion of water hyacinth into second generation ethanol reduces the problem of ecosystem imbalance, however, the process requires the best pretreatment and hydrolysis techniques.

Pretreatment using acid (H2SO4, HNO3, HCl), alkaline (NaOH), heat or a combination has been studied and the best lignin removal involves the use of H2SO4 at high temperatures [7,12], however, the use of NaOH is better than H2SO4 or H2O2 [5]. Nonetheless, the use of alkaline or acidic pretreatment methods have their individual weakness and advantages.

Fungi are well-known to effectively decompose cellulose organic matter. Implementing T. reesei as a biological catalyst for producing cellulose enzyme in alkaline treated water hyacinth, produced 0.531 g/g of sugar [13] whereas, the use of Aspergillus niger produced sugar as much as 13.5 g/L [5]. It also produces various enzymes that are able to degrade polysaccharides in plant cell walls [13]; furthermore, it is one of the most commonly encountered saprophytic fungi in the air which plays an important role in the recycle of carbon and nitrogen globally. A. fumigatus generally is a terrestrial fungus which lives on dead or decayed materials, it can also spread through the air as an asexual spore [14] and it is able to produce cellulose enzymes needed in the hydrolysis process [7].

This study objective therefore, is to utilize water hyacinth as a lignocellulose-containing biomass applying A. fumigatus fungi as a biocatalyst in the ethanol production process. The results are expected to provide an alternative to solve the problem of imbalance within the aquatic ecosystem which is caused by the presence of weeds.

2. Research methodology

2.1. Materials and microorganism
Water hyacinth was obtained from the Citarum River, West Java, this was then washed to remove dirt and cut to a size of 2-2.5 mm, then dried in an oven at 100°C for 5-6 hours. The dried water hyacinth was mashed with a blender and sifted to form a powder with a 25 mesh and then stored at room temperature until it was used as a substrate, ready to be delignified [1]. A. fumigatus was obtained from the Laboratory of Environmental Biology/Microbiology, Department of Environmental Engineering, Universitas Trisakti, Jakarta, Indonesia. It was cultivated on potato dextrose agar (PDA) as growth media for 5 days at 30°C (until perfect sporulation was formed), this was thereafter stored at 40°C until it was utilized [7].

2.2. Pretreatment of water hyacinth
Pretreatment was carried out using A. fumigatus fungi as a source of enzymes, fungi and substrates were put into containers containing potato dextrose broth (GDP) (sterile autoclave at 121°C and 15 PSI, for 15 minutes) with a ratio of fungi to substrate varied at 1: 1, 1: 5, and 1:10 (w / w). Furthermore, observations were made at the contact time of 1 day, 3 days and 5 days.

2.3. Enzymatic hydrolysis
Enzymatic hydrolysis is carried out using cultivated and dried A. fumigatus, this was then added to the substrate at a ratio of 1: 1, 1: 5, and 1:10 (w / w) with contact time of 1 day, 3 days and 5 days. Thereafter, it is filtered and the filtrate is stored and ready for use.
2.4. Analytic methods
Lignin examination was carried out using the gravimetric method. The lignin removal efficiency can be calculated using equation 1.

\[
\text{Lignin removal efficiency (\%)} = \frac{W(a) - W(b)}{W(a)}
\]  

(1)

\(W(a)\) : Lignin level before delignification (\%)

\(W(b)\) : Lignin levels after delignification (\%)

The estimated sugar produced at the hydrolysis stage was analyzed using the DNS method [14], its reagents were prepared by adding 1 gram of DNS, 50 mg of sodium sulfite and 1 gram of NaOH. All three were dissolved in 50 mL of distilled water placed in a 50 mL colored flask (this is to avoid oxidation). The estimation of sugar was carried out by adding 3 mL of sample and 3 mL of DNS reagent to the test tube, which was then closed with aluminum foil and further heated at 90°C for 15 minutes or until the color turned brownish red. An aliquot (1 mL) of Rochelle salt which was prepared by dissolving 20 grams of Tartar Na-K in a 50 mL volumetric flask was then measured by a spectrophotometer at a wavelength of 575 nm.

3. Results and discussion

3.1. Effect of pretreatment
Separation of lignin and carbohydrates is needed to facilitate the hydrolysis process during the produce of sugar. Table 1 shows the allowance for lignin content while figure 1 shows the allowance for lignin content on the substrate.

| Ratio of fungi: substrat | Contact time (hours) | Lignin removal efficiency (\%) |
|-------------------------|----------------------|--------------------------------|
| 1 : 1                   | 24                   | 22.6                           |
|                         | 72                   | 25.9                           |
|                         | 120                  | 20.6                           |
| 1 : 5                   | 24                   | 15.6                           |
|                         | 72                   | 21.9                           |
|                         | 120                  | 17.8                           |
| 1 : 10                  | 24                   | 8.3                            |
|                         | 72                   | 13.2                           |
|                         | 120                  | 6.4                            |

The best delignification process was found in the 1:1 ratio of fungi to substrate at the contact time of 3 days with the allowance of 25.9%. Figure 1 shows that at each contact time variation the ratio of 1:1 has the highest efficiency. These results indicate that *A. fumigatus* is able to degrade the lignin compounds contained in the substrate effectively at this ratio.

![Figure 1. Allowance lignin content of water hyacinth.](image-url)
Allowance efficiency as shown in equation 1, indicates that it is lower than that shown by the researcher [15], who stated that the best delignification involves the use of H₂O₂. Furthermore Singh and Bishnoi, states that the use of sodium hydroxide is able to exclude up to 56% lignin content by [5]. The latter was however chosen because it is a reagent that can remove lignin without affecting other components [1]. Thus, the use of biological catalysts is more environmentally friendly and does not cause side effects in the process.

3.2. Hydrolysis process

After undergoing the delignification stage, the biomass is then processed to produce sugar through hydrolysis process, whose fermentation precedes the production of bioethanol and hydrolysis carried out using alkali, acids or enzymes.

![Figure 2. Sugar production on hydrolysis stage.](image)

**Table 2.** Sugar production on hydrolysis process.

| Ratio of fungi : substrat | Contact time (hours) | Glucose (g/L) |
|--------------------------|----------------------|---------------|
| 1 : 1                    | 24                   | 0.32          |
|                          | 72                   | 0.32          |
|                          | 120                  | 0.31          |
| 1 : 5                    | 24                   | 0.50          |
|                          | 72                   | 0.25          |
|                          | 120                  | 0.12          |
| 1 : 10                   | 24                   | 0.06          |
|                          | 72                   | 0.02          |
|                          | 120                  | 0.12          |

In this study, enzymatic hydrolysis produced the highest sugar (0.5 g / L) at the ratio of 1: 5 in 24 hours contact time. However, the result was similar at a ratio of 1: 1 with a 24 hour contact time as shown in Table 2 and Figure 2. *A. fumigatus* in this research is able to produce cellulose enzymes needed in the hydrolysis process. These results are lower than those indicated when water hyacinth was treated earlier with bases ie 13.5 g/L [5], 0.531 g/g [16], 0.41 g/g and with initial acid treatment [17], 0.5672 g / g [12], and 396.6 mg/g [11].

4. Conclusion

This study proves that *A. fumigatus* functions as a biocatalyst suitable in the delignification process as a pretreatment to the hydrolysis process. The addition of *A. fumigatus* to the substrate at a ratio of 1: 1 in the delignification process is the most applicable when compared with others, this is because lignin removal occurred optimally at this proportion, which is 25.9% at 72 hours contact time. Furthermore, the highest sugar level of 0.5 mg/L was observed at a ratio of 1: 5 with a contact time of 24 hours. The
fermentation phase needs to be carried out to further investigate the potential of water hyacinth as a raw material for ethanol production.

References

[1] Ganguly A, Chatterjee P K and Dey A 2012 Studies on ethanol production from water hyacinth: A review Renewable and Sustainable Energy Reviews 16(1) 966-972
[2] Guragain Y N, Coninck J D, Husson F, Durand A, Rakshit S K 2011 Comparison of some new pretreatment methods for second generation bioethanol production from wheat straw and water hyacinth Bioresour. Technol. 6 4416–4424
[3] Rezania S, Mohd F M D, Shaza E M, Johan S, Shazwin M T, Mohd B M Y, Nesam K, Negisa D, Amimul A 2017 Review on Pretreatment Methods and Ethanol Production from Cellulosic Water Hyacinth BioResources 12(1) 2108-2124
[4] Ahn D J, Kim S K and Yun H S 2012 Optimization of pretreatment and saccharification for the production of bioethanol from water hyacinth by Saccharomyces cerevisiae Bioprocess Biosyst Eng 35 35–41
[5] Singh A and Bishnoi N R 2013 Comparative study of various pretreatment techniques for ethanol production from water hyacinth Industrial Crops and Products 44 283-289
[6] Xia, Ao, Jun C, Wenlu S, Cong Y, Junhu Z and Kefa C 2013 Enhancing enzymatic saccharification of water hyacinth through microwave heating with dilute acid pretreatment for biomass energy utilization Energy 61(2013) 158-166
[7] Das A, Gosh P, Paul T, Gosh U, Pati B R and Mondal K C 2016 Production of bioethanol as useful biofuel through the bioconversion of water hyacinth (Eichhornia crassipes) Biotech 670
[8] Bayarakci A G and Kocar G 2014 Second-generation bioethanol production from water hyacinth and duckweed in Izmir: a case study Renew. Sustain. Energy Rev. 30 306-316
[9] Bhatia L, Johri S and Ahmad R 2012 An economic and ecological perspective of ethanol production from renewable agro waste: a review AMB Express 2 65
[10] Ogeda T L and D F S Petri 2010 Hidrólise enzimática de biomassa Química Nova 33 1549–58
[11] Mohapatra P, Padhy S, Das Mohapatra P K and Thatoi H N 2018 Enhanced reducing sugar production by saccharification of lignocellulosic biomass, Pennisetum species through cellulase from a newly isolated Aspergillus fumigatus Bioresource Technology
[12] Zhang Q, Weng C, Huang H, Wang V A D 2015 Optimization of Bioethanol Production Using Whole Plant of Water Hyacinth as Substrate in Simultaneous Saccharification and Fermentation Process Frontiers in Microbiology 6 1411
[13] Paulusse C, Hallsworth J E, Álvarez-Pérez S, Nierman W C, Hamill P G, Blain D, Rediers H, Lievens B 2016 Ecology of aspergillosis: insights into the pathogenic potenicy of Aspergillus fumigatus and some other Aspergillus species Microbial Biotechnology 10(2) 296–322
[14] Miller G L 1959 Use of dinitrosalicylic acid reagent for determination of reducing sugar Anal. Chem. 31 426–428
[15] Manivannan A and Narendhirakannan R T 2015 Bioethanol Production From Aquatic Weed Water Hyacinth (Eichhornia crassipes) by Yeast Fermentation Springer Science Business Media Dordrecht
[16] Pothiraj C, Arumagam R and Gobinath M 2014 Sustaining ethanol production from lime pretreated water hyacinth biomass using mono and co-cultures of isolated fungal strains with Pichia stipitis Bioresource and Bioprocessing 1(27)
[17] Das S, Bhattacharyya A, Haldar S, Ganguly A, Ting Sai Gu Y P and Chatterjee P K 2015 Optimization of enzymatic saccharification of water hyacinth biomass for bio-ethanol: Comparison between artificial neural network and response surface methodology Sustainable Materials and Technologies