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Screening of Factors Affecting Biological Delignification Process of Oil Palm Trunk Using Local Oyster Mushroom (*Pleurotus Ostreatus*)

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Abstract

Oil palm trunk, a lignocellulosic waste, contains high cellulose content. The removal of lignin is essential as lignin inhibits almost all reactions occur on cellulose. This study is about the usage of a local oyster mushroom (*Pleurotus ostreatus*) as an agent of delignification and the screening of various factors in order to achieve the highest amount of lignin removal. The software Design-Expert version 6.08 is used to determine the most relevant design for the screening process which leads to the method of two-level factorial design with seven parameters at 1/8 fraction. Sixteen runs were done for the purpose of studying the effect of temperature (20°C and 30°C), pH value (5 and 8), humidity (controlled by the presence of silica gel), light exposure, moisture (controlled by the volume of water added, which varies from 0.5 mL to 5.0 mL), the ratio of fungi to medium (1:2 and 1:10) and contact time (from 2 and 10 days) in which all the respective values were obtained from literature study. From the analysis, the main effects of the process are temperature, which contributes up to 32.20%, pH value, which contributes up to 10.08%, fungi to the medium ratio which contributes up to 8.82% and moisture content, which contributes up to 7.63%. This study also found that there are interactions between main effects, which are between temperature and pH value, and temperature and fungi to the medium ratio. These findings proved that each of the factors may affect one and another during the process.

Keywords: lignocellulose, oil palm trunk, biological delignification, lignin removal, oyster mushroom, *Pleurotus ostreatus*, two-level factorial design, Design-Expert

1. Introduction

Malaysia is one of the largest contributors of palm oil production. The combined oil palm production of Malaysia and its neighboring country, Indonesia contributes 88% of the world palm oil production [1]. Palm oil based product includes detergents, frying oil, cosmetics and even food product such as margarine. Palm oil potential on bioethanol production has also grown throughout the years. It has been used in recent researches as a material for bioethanol production [2][3].

An oil palm tree has a time span of around 20 to 25 years. After this time span, the oil palm tree will lose its ability to produce the sufficient amount of palm oil. Usually, it will be cut down for the next replantation of the new oil palm tree. The old oil palm tree will be used for different purposes depending on the tree parts. Oil palm trunk is one of the parts on the oil palm tree which usually has been used to make plywood in the logging industry. However, at the core of the trunk, it is actually soft and contains up to 80% of sap, which makes that part not a suitable choice of source to make plywood [4]. After the sap is squeezed from the trunk, the fiber wastes of the trunk left after the process usually will be used as fertilizer. However, the usage of these wastes as fertilizer is still in question as it yields traces of the sap plus it contains a high composition of cellulose. The potential of these wastes as a raw material for cellulose-based product is high. In a way, these wastes serve as a new source for bioethanol production from lignocellulosic material.

1.1 Selection of Delignification Method

In order to remove the lignin from cellulose, delignification process is required. There are various methods of the delignification process which is also known as the lignin removal process. From the simplest method to the most complicated ones, these methods have their own advantages and disadvantages. The method used usually will be determined depending on the type of feedstock used.

Physical method is a method of delignification, which favors the physical treatment applied to the feedstock. This method is focused on the destruction of the lignin structure in order to allow any reaction to take place to the cellulose. Chipping, grinding and milling are all categorized under physical treatment. This may seem as the simplest method of delignification. However, this method requires high-energy consumption and the equipments used for the process is high in cost, making the capital cost of the method quite high. Equipments such as sawing machines and grinders will also require the specific expertise which will only increase the manpower needed, increasing the operating cost [12].

Another well-known delignification method in the bio-energy industry is the chemical method. As its name proposed, the chemical method focuses on the usage of chemical in order to break the bonds of lignin structure in order to react with cellulose. The usual chemical method is by using the dilute-acid hydrolysis or concentrated-acid hydrolysis processes. Other types of similar hydrolysis method do share the same concept of degrading the lignin structure. However, the high cost of the chemical used in this procedure seems to be the drawback of this method as the chemical used scales with the amount of the feedstock [12]. Another drawback of this method is that the chemical used to have a tendency of degrading the cellulose during the process if the process is poorly supervised.

Biological method, on the other hand, is not a new delignification method but the method itself hasn't been applied widely in a large-scale production, mainly because of its time-consuming process compared to other methods. However, from other aspects such as the economy and energy cost, it shows the most potential to be the leading method in delignification. The biological method uses other microorganisms as an agent for the delignification process such as fungi. Known fungi, which have shown positive results on the delignification are such as the white-rot, soft-rot and brown-rot fungi [5][6]. However, brown-rot and soft-rot fungi show the tendency to degrade cellulose during the degradation of lignin. Only white-rot fungi have shown the positive results. Other benefits of biological method include the production of enzymes, which will be used in the latter stage of the process in the production of bioethanol.

1.2 Selection of Fungi

As explained in the previous section, white-rot fungi are the common candidate to be used as an agent of delignification. Their ability to remove lignin without severely damaging the cellulose during the process is their difference compared to other types of fungi. However, this type of fungi has a wide range of species making it harder to choose the best candidate for delignification process.

Known white-rot fungi that have been used in researches for lignin removal purposes are such as *Phanerochaete chrysosporium*, *Trametes* (*Coriolus*) *versicolor*, *Ceriporiopsis subvermispora*, and *Pleurotus ostreatus* [13]. From the study, *T. versicolor* shown as the most efficient lignin remover as from the Klason lignin determination method, but due to the low amount of cellulose left in the end of the experiment, it is shown that this fungus does not conserve cellulose during the lignin removal. *P. ostreatus* on the other hand, has the second-lowest amount of lignin with the highest cellulose amount. *C. subvermispora* and *T. versicolor* seems to be degrading cellulose rather than lignin. As for this study, *P. ostreatus* is selected as it shows the most consistent yield of all the white-rot fungi [13]. As an addition, the *P. ostreatus* is also a common local delicacy in Malaysia making it easier to obtain compared to other fungi.

1.3 Experimental Design

The design of experiment for this study is determined using Design-Expert version 6.08. Design-Expert is a software that aids researchers in design of experiment. Often used for screening and optimization processes, this software will propose the most significant design for an experiment according to the type of the process. However, the limitation of this software is that the proposed experiment is designed relevantly according to the parameters entered. If the parameters entered for the design is flawed, it will propose an experimental design which will not result in the most efficient result. In other to avoid such scene, the parameters chosen or range value selected in this study were first studied from other literature that were relevant to the study.

To identify the factors that will affect the delignification process, the growth of the fungi is monitored. This is due to as the fungi grow; it will continuously degrade lignin as a source of energy, hence making the fungi growth factor and the delignification factor identical. The factors which will affect the delignification process should be the significantly same factors that affect the growth of the fungi. Temperature, pH value, humidity, darkness, medium moisture content, contact time and fungi to the medium ratio are the controlled factors in this experiment. Each of these factors was studied at a certain range of values based from the literature study [5][7][8].

The purpose of this study is to determine the main effects that would affect the biological delignification of oil palm trunk waste. Seven parameters were studied of their effect to the treatment process using specific screening method. The software Design-Expert is used in order to determine the most suitable screening method.

Another purpose of this study is to study the interactions between main effects. This study is crucial for optimization because each of the main effects had the tendency to affect one another as the experiment runs. For this study, Design-Expert is also used to determine the interaction between the main effects.

2. Materials and Method

In this study, the oil palm trunk used was obtained from a local oil palm plantation located near Jerantut, Pahang. The sap from the oil palm trunk was squeezed and peeled leaving only the fiber which was then stored in a freezer. These fibers were dried at 60°C in an oven for eight hours or until the weight is constant before it was used in the study.

2.1 Screening Method

With the usage of Design-Expert, the screening method suitable for these parameters is the two-level factorial design with seven parameters. This study was done at 1/8 fraction, which leads to 16 runs of experiments with various parameter's values. By using this method seven of the parameters were screened to determine which of the parameters are the main factor and the interaction between them. This screening process was used to determine which factor gives the most contribution to the process besides determining the interaction between the factors. This method will also determine the optimized range of value for each parameter.

2.2 Preparation of Fungi

As for this research, a local type of fungi, *Pleurotus ostreatus* or commercially known as "Oyster Mushrooms", were used as an agent of delignification. These fungi spores were obtained from a local established commercial mushroom supplier. The part that contains the most mushroom spores is the bottom surface of the canopy. The bottom surface of the canopies was extracted from the rest of the fungi to obtain as many spores as possible. These parts were cut into pieces of around 5 cm length before it was weighed to 1 gram of portions. These portions will be used to breed fungi on the medium inside the reactor. The equipments used for this process was carefully sterilized to avoid any contamination.

2.3 Experimental Setup

The reactor used is 250 mL of bottles with different properties depending on the runs. In this research, there are two effects or factor that falls under categorical factor. Lighting and humidity were studied at two fixed point. Lighting was controlled by wrapping the reactors with aluminum foils to adjust the light and dark properties. Humidity was controlled by the presence of silica gels inside the reactors. The other five factors were all numeric factors where these factors were studied in their range of value respectively. Fungi to the medium ratio was controlled by the weight of the fungi and medium. Fungi was inserted into the reactor after the medium is in place. The samples were then sterilized by autoclave. The pH values of the samples were controlled using citric buffer solutions. The buffer solutions were added to the medium just before the samples were placed into the incubator. The temperature was controlled by the incubator temperature controller. Medium moisture content was controlled by the volume of water added to the samples for each 12 hours. Contact time was controlled by removing the fungi from the medium at a specific time. These seven parameters were controlled and observed for the screening process. Table 1 shows the condition and value of each parameter for the process.

2.4 Analysis Method

The analysis of the lignin degraded is determined by the Klason lignin determination method [9]. This method uses high concentrated sulphuric acid to degrade compounds in the medium other than lignin. After the contact time of each sample, the medium was washed thoroughly in order to remove any fungi and in order to neutralize the pH value of the medium. The mediums were then dried in an oven at 60°C for eight hours or until the weight is constant before it was treated with the Klason lignin determination method. One gram of the sample was taken out and placed into a 100 mL beaker before adding 20 mL of 72% sulphuric acid and left at room temperature for two hours. After two hours, 560mL of distilled water were added to the sample and were heated at 100°C in shaking water bath for another two hours. The samples were then washed and filtered before it was dried in an oven at 60°C for eight hours or until the weight is constant before they were left in decicator for at least two days until the weight is constant. The final weight of each sample is the weight of the lignin left in the samples respectively and were recorded as the result of biological delignification.

Table 1. Condition and Values of Each Parameters for the Delignification Process

Run	Temperature (°C)	pH Value	Humidity (Silica gel packet)	Lighting	Moisture (mL of water added per 12 hours)	Fungi to Medium ratio	Contact Time (days)
1	20	5	None	Dark	0.5	1:10	2
2	30	5	None	Dark	5.0	1:2	10
3	20	8	Yes	Dark	0.5	1:2	10
4	30	5	Yes	Light	0.5	1:10	10
5	30	8	Yes	Dark	5.0	1:10	2
6	30	8	None	Light	0.5	1:2	2
7	20	8	None	Light	5.0	1:10	10
8	20	5	Yes	Light	5.0	1:2	2
9	20	5	None	Light	0.5	1:2	10
10	30	5	None	Light	5.0	1:10	2
11	20	8	Yes	Light	0.5	1:10	2
12	30	5	Yes	Dark	0.5	1:2	2
13	20	8	None	Dark	5.0	1:2	2
14	30	8	Yes	Light	5.0	1:2	10
15	20	5	Yes	Dark	5.0	1:10	10
16	30	8	None	Dark	0.5	1:10	10

3. Results and Discussions

3.1 Main Effects Analysis

From Fig. 1, it is shown that parameter A, temperature, contributes the most to the delignification with a percentage contribution as much as 32.20%. Reference [10] stated that for the cultivation of *Pleurotus ostreatus* is actually depended on the surrounding temperature of the medium. According to the research, the temperature varies depending on the type of the fungi and the contact time. At different contact time, different processes occur between the fungi and the medium. The temperature value may also affect the activity of the lignin peroxidase which is released by the fungi.

The second factor that contributes to the delignification is the pH value during the growth with the contribution as much as 10.08%. Fungi tends to release different type of enzymes depending on the pH value of its surrounding. At a different level of pH value, different enzymes will be released and as the delignification process is greatly affected by lignin peroxidase enzymes, pH value plays a major role in the delignification [11].

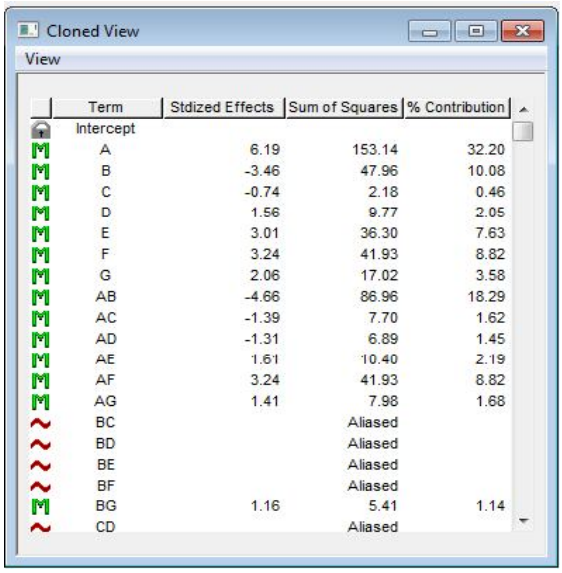
Fungi to the medium ratio contributes as much as 8.82% to the delignification process. The amount of fungi presents on the medium seem to affect the delignification itself as this contribution shows that there is a limited amount of medium that can be treated by the fungi. Higher presence of fungi does not give a higher delignification results and higher amount of medium seems to be a bit too handful for the fungi to treat.

Moisture content of the medium contributed as much as 7.63% to the delignification process. This factor seems to show its contribution to the process as these fungi are naturally favored on growing at wet habitats.

Contact time is one of the factors that gave as low as 3.58% of contribution to the delignification process. From the result, this factor might not be one of the most affecting factors hence. It will be fixed into one fixed value for the optimization process. Although the biological delignification process is actually a process which is time consuming, the contact time for this process has shown to be less important compared to other factors.

The lighting during the delignification has also proven to be less affective. The lighting is actually critical for oyster mushroom breeding but for delignification, it shows such low effect. With a percentage of contribution as low as 2.05%, it will be set to a fixed value in the next optimization step.

The least effective factor is the humidity which contributes 0.46%. The presence of silica gel in the process has proven to be useless as these fungi will still degrade lignin at the studied humidity level. In the oyster mushroom breeding, humidity was never properly controlled, and this result has shown that for it is not a factor that may give a huge effect to the biological delignification.



Term	Stdized Effects	Sum of Squares	% Contribution
Intercept			
A	6.19	153.14	32.20
B	-3.46	47.96	10.08
C	-0.74	2.18	0.46
D	1.56	9.77	2.05
E	3.01	36.30	7.63
F	3.24	41.93	8.82
G	2.06	17.02	3.58
AB	-4.66	86.96	18.29
AC	-1.39	7.70	1.62
AD	-1.31	6.89	1.45
AE	1.61	10.40	2.19
AF	3.24	41.93	8.82
AG	1.41	7.98	1.68
BC		Aliased	
BD		Aliased	
BE		Aliased	
BF		Aliased	
BG	1.16	5.41	1.14
CD		Aliased	

Figure 1. The percentage contribution of each main factors and their interaction during the experiment to the delignifcation. Terms: A, temperature; B, pH value; C, humidity; D, lighting; E, moisture content; F, fungi to medium ratio; G, contact time

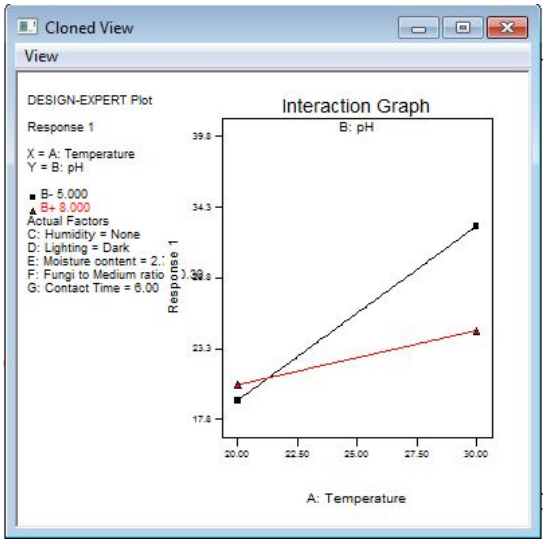


Figure 2. The interaction graph between factor A, temperature and factor B, pH value

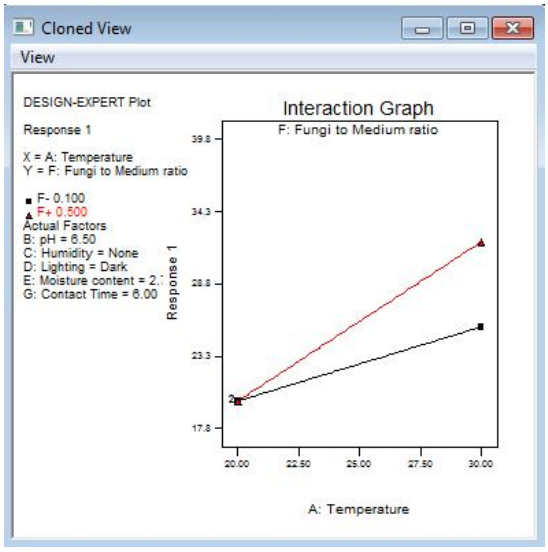


Figure 3. The interaction graph between factor A, temperature and factor F, fungi to medium ratio

2.5 Interactions between Factors

Between these factors, there are interactions that will be likely to improve the delignification process. Some factor interactions may contribute more than the main effects alone. There are two interactions discovered in this study.

The first interaction is between factor A, temperature and factor B, pH value. Fig. 2 shows that there exists an interaction between temperature and pH value. This interaction is possibly due to both factors contribute the most compared to other factors. The pH value contributes to the release of lignin peroxidase enzymes, which degrades lignin upon release. However, these enzymes will be most active in a certain range temperature range, thus, degrading more lignin [11]. This shows that these two factors have the interaction between them that affect the delignification of the medium.

The second interaction can be found in Fig. 3, which shows the interaction between factor A, temperature and factor F, fungi to the medium ratio. The high contribution of temperature in the process has already been explained for a few times [10][11], but its interaction with another factor, fungi to the medium ratio, is something worth discussing. The fungi to medium ratio factor give a significant amount of contribution to the process but not as much as pH value. This may go back to the lignin peroxidase enzymes released by the fungi. At a high amount of fungi compared to medium, the more lignin peroxidase will be released but the temperature will still be affecting the activity of the enzymes hence showing that these two main effects have a unique interaction between them.

4. Conclusions

From this study, it is to be concluded that in the delignification of the oil palm trunk using oyster mushrooms, temperature contributes the most to the delignification process which is as much as 32.20% followed by pH value at 10.08%, fungi to the medium ratio at 8.82%, moisture content at 7.63%, contact time at 3.58%, lighting at 2.05% and the least affecting factor, humidity at 0.46%. The two interactions between temperature and pH value, and temperature with fungi to the medium ratio proved that these factors may affect one another's effect to the process. The two keys of delignification using oyster mushrooms are the fungi growth condition and the activity of lignin peroxidase enzymes.

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