Factorial analysis of mycelium growth in *Pleurotus* sp. cultivation by using agricultural wastes

N A Dzulkeflı\(^1\) and N Zainol\(^2\)*

\(^1\)Faculty of Chemical & Natural Resources Engineering, Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.
\(^2\)Centre of Excellence for Advanced Research in Fluid Flow (CARIFF), Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia.

*azwina@ump.edu.my

**Abstract.** Oyster mushrooms (*Pleurotus ostreatus*) have three stages of mushroom growth, which are mycelium growth (spawn running), pinhead initiation and fruiting body development. Rubber sawdust is commonly used as a media for oyster mushroom cultivation. However, the low availability of rubber trees has become a serious problem to the mushroom’s grower. The purpose of this study is to evaluate the factors affecting mycelium growth in *Pleurotus* sp. cultivation by using agricultural wastes. Two different substrates were used namely, empty fruit bunch (EFB) and sugarcane bagasse (SB). The other selected factors were size of substrates (0.5 cm and 2.5 cm), mass ratio of spawn to substrate (SP/SS) (1:10 and 1:14), temperature (25°C and ambient) and pre-treatment of substrates (steam and non-steam). The responses were mycelium extension rate (M) and nitrogen concentration in mycelium (N). Design Expert software was used to conduct experimental design where all the factors were randomized. As a result, pre-treatment and type of substrate were the most contributing factors on both M and N, respectively. It can be concluded that the agricultural waste such as empty palm fruit bunch (EFB) can replace the sawdust as a media for oyster mushroom cultivation.

1. Introduction

Oyster mushroom is belong to the genus of *Pleurotus* [1]. Mushroom growth has 3 stages which are mycelium growth (spawn running), pinhead formation and fruiting body development [2]. The growth stage of fungal mycelium in the substrate is very important for mushroom production, since suitable substrate facilitate mycelium colonization and avoid risks of contamination during fruiting body development [3]. It also creates suitable internal conditions for fruiting in order to have high yield of oyster mushroom [4]. Mushroom requires carbon, nitrogen and inorganic compounds especially at the stage of mycelium growth as its nutritional sources for growth. Nitrogen is an essential element required by all fungi which is composed of β(1-4)-linked unit N-acetylglucosamine [5]. Neelam et al. [6] proved that the ammonium chloride is the major component of nitrogen in the substrate that supported the growth of mycelium of *P. florida* and *P. ostreatus*. Ortega et al. [7], in their studies with *Pleurotus* sp. described a nitrogen increase in mushroom mycelium related to the amount of nitrogen presented in the initial substrate plus the nitrogen amount in the inoculums.

In the context of Malaysia, even though the oyster mushroom cultivation has a good market potential but the fresh stocks of fresh mushrooms in the market are still inadequate to meet local demands [8]. Rubber sawdust is commonly used in Malaysia as a media for oyster mushroom cultivation. However, the low availability of rubber tree has become a serious problem to the
mushroom’s grower. Thus, the new alternative substrates need to be used to overcome the shortage of mushroom production [9]. Sugarcane waste, paddy straw and tea waste [10] can be used as the new alternative substrate. In order to find the new alternative substrates for *Pleurotus* sp. cultivation, there were a few factors that contributed in the cultivation such as the type of substrate, pH of substrate, moisture content, temperature, size of substrates, mass ratio of spawns to substrates and pre-treatment of substrate [11]. The objective of this study is to evaluate the factors affecting the mycelium growth in *Pleurotus* sp. cultivation by using agricultural wastes (empty palm fruit bunch (EFB), coconut fiber (CF), banana stem (BS), sugarcane bagasse (SB), coffee ground (CG) and egg trays (ET)). The factors were type of substrate (SB-A and EFB-B), size of substrates (0.5 cm and 2.5 cm), mass ratio of spawn to substrate (SP/SS) (1:10 and 1:14), temperature during spawn running (25°C and ambient) and pre treatment of substrates (steam and non steam). The most contributing factors and interaction between the factors were analyzed via two level factorial analysis (TLFA). Please state the brief findings/outcomes from this study here.

2. Materials and Methods

2.1. Collection of substrates and spawns

The empty palm fruit bunch (EFB) as a substrate was collected from palm oil plantation in Kuala Selangor, coconut fiber (CF) was collected from coconut plantation in Kuala Selangor and banana stem (BS) was obtained from banana farm at Banting, Selangor. Meanwhile, the sugarcane bagasse (SB) was collected at Semenyih, Selangor and coffee ground (CG) as well as egg trays (ET) were domestic wastes from Bangi, Selangor.

2.2 Preliminary experiment

During first preliminary experiment, the oyster mushroom was cultivated by using five different substrates which were EFB, CF, ET and CG. The EFB, CF and ET were soaked in the water for 12 hours [12] without cutting into pieces. Meanwhile, the CG was soaked in water for 30 minutes. Then, the substrates were filtered to drain excess water [4]. The substrates were mixed with oyster mushroom spawn. For BS, two holes were made for 1 inch of depth and a few spawns were put in each holes [13]. All substrates were left in the dark condition at ambient (28 to 30°C) temperature until the substrates were fully colonized with mycelium.

During the second preliminary experiment, ET was changed to SB substrate. Hundred gram of EFB, SB and BS were cut into 2.5 cm and soaked in water for overnight. Meanwhile, the CG was soaked in water for 15 min and drained the excess water [14]. The substrates were mixed with oyster mushroom spawn and left in the dark condition at ambient temperature (please specify) until the substrates were fully colonized with mycelium.

During the third preliminary experiment, only SB substrate was prepared and inoculated with the oyster mushroom spawn in order to measure the mycelium growth extension (M). The SB was prepared as second preliminary experiment and the spawn was placed on the surface of substrate so that the mycelium spreading from the surface towards the bottom of the container [4].

From the preliminary experiment, two substrates were chosen for factorial analysis experiment based on the highest mycelium extension rate (M).

2.3 Experimental set-up for factorial analysis

There were five selected factors that gave contribution to oyster mushroom growth (Table 1). The factors involved were type of substrates, size of substrates, mass ratio of spawn to substrates (SP/SS), temperature and pre-treatment of substrates. SB (A) and EFB (B) were prepared in bottles according to run in Table 2. Firstly, the substrates were cut into the selected size, then soaked in water for overnight, filtered to drain excess water and weighed for 100 g. Then, the substrates were pre treated with selected pre treatment and inoculated with spawn by placing the spawn on the surface of substrate. The bottles were closed and incubated at selected temperature in the dark condition. The
experiment was conducted according to set-up in Table 2. The experiment set-up in Table 2 was performed by Design Expert software where all the factors were randomized [15]. Then, the experimental data were analyzed by using Design Expert software in order to determine the most contributing factors. There were two responses which were mycelium extension rate (M) and nitrogen concentration in mycelium (N).

Table 1. Selected factor and their range.

| Factors                        | Low level | High level |
|--------------------------------|-----------|------------|
| Type of substrate              | A         | B          |
| Size of substrates             | 0.5 cm    | 2.5 cm     |
| SP/SS                          | 1:10      | 1:14       |
| Temperature during spawn running | 25°C     | Ambient   |
| Pre-treatment of substrates    | Steam     | Non-steam  |

Table 2. Experimental set-up that has been constructed by using two level factorial analysis (TLFA) Design Expert software (Version 7).

| Run | A: Type of substrate | B: Size of substrates (cm) | C: SP/SS (g/g) | D: Temperature | E: Pre-treatment of substrate |
|-----|----------------------|-----------------------------|----------------|----------------|-------------------------------|
| 5   | A                    | 0.5                         | 1:10           | 25 °C          | Steam                         |
| 8   | B                    | 0.5                         | 1:10           | 25 °C          | Non-Steem                     |
| 9   | A                    | 2.5                         | 1:10           | 25 °C          | Non-Steem                     |
| 14  | B                    | 2.5                         | 1:10           | 25 °C          | Steam                         |
| 3   | A                    | 0.5                         | 1:14           | 25 °C          | Non-Steem                     |
| 13  | B                    | 0.5                         | 1:14           | 25 °C          | Steam                         |
| 4   | A                    | 2.5                         | 1:14           | 25 °C          | Steam                         |
| 2   | B                    | 2.5                         | 1:14           | 25 °C          | Non-Steem                     |
| 15  | A                    | 0.5                         | 1:10           | Ambient        | Non-Steem                     |
| 7   | B                    | 0.5                         | 1:10           | Ambient        | Steam                         |
| 16  | A                    | 2.5                         | 1:10           | Ambient        | Steam                         |
| 11  | B                    | 2.5                         | 1:10           | Ambient        | Non-Steem                     |
| 6   | A                    | 0.5                         | 1:14           | Ambient        | Steam                         |
| 12  | B                    | 0.5                         | 1:14           | Ambient        | Non-Steem                     |
| 1   | A                    | 2.5                         | 1:14           | Ambient        | Non-Steem                     |
| 10  | B                    | 2.5                         | 1:14           | Ambient        | Steam                         |

2.4 Validation experiment
Validation experiment was conducted based on the suggested of the best conditions from Design Expert software. The criteria set-up to select the best processing conditions were given in Table 3. All factors were set as in range. Meanwhile, the responses were set to get the maximum value.
Table 3. Criteria for validation experiment.

| Name                              | Goal         | Value          |
|-----------------------------------|--------------|----------------|
| Type of substrate                 | Is in range  | A - B          |
| Size of substrate                 | Is in range  | 0.5 cm - 2.5 cm|
| SP/SS                             | Is in range  | 1:10 - 1:14    |
| Temperature                       | Is in range  | 25°C - Ambient |
| Pre treatment of substrate        | Is in range  | Steam - Non steam |
| Mycelium extension rate (M)       | Maximize     | -              |
| Nitrogen concentration (N)        | Maximize     | -              |

2.5 Sample analysis

Sample analysis was conducted after all run fully colonized with mycelium. There were two responses, which were mycelium extension rate (M) and nitrogen concentration in mycelium (N).

2.5.1 Determination of mycelium growth.

Spawn run (mycelium extension) was observed regularly until white colonization appeared. Each experiment was determined based on the days for mycelium growth to complete. Mycelium growth was measured in centimeter as the length of the mycelium spreading from the surface of substrates toward the bottom of bottles [4].

2.5.2 Nitrogen concentration analysis by using HACH Spectrophotometer.

N was determined by using Persulfate Digestion Method (Method 10072). The mycelium was collected from the substrates and diluted by using deionized water using 40 dilution factor. Then, N was analyzed by using HACH Spectrophotometer.

2.6 Data analysis

All data obtained were recorded in Design Expert software. The responses were analyzed using Analysis of Variance (ANOVA) based on p-value with 95% of confidence level to identify the most contributing factors and interaction between the factors that has an effect on both responses M and N.

3. Results and discussions

3.1 Preliminary experiments

Figure 1 shows the mycelium growth in the substrates. After 12 days, EFB and CF showed high amount of mycelium (red arrows) but other substrates showed less mycelium growth in the substrates. This may be due to ability of EFB and CF retain moisture content for long period of time [16]. According to Chang and Miles [17], the appropriate moisture in the substrate should encompass a range between 50% and 75% in the substrate, enabling the satisfactory growth of *Pleurotus* sp. mycelium. However, both seemed to have longer time to fully colonized because the substrates were not cut into smaller size, so the surface area of the substrates were lower. Substrates need to be cut into 2-6 cm to provide a larger surface area, thus improving the oxygen transfer to the substrate [18]. Besides, the mycelium in both BS and ET increased in the early days but started to decrease after 9 days. This may be due to the loss of moisture content of the substrates and low mass ratio of spawn to substrates. Therefore, the spawn could not obtained enough nutrients from the substrates because of the low moisture content in the substrate the resulted in difficult breathing for the mycelium [11].
Figure 1. First preliminary experiment after 12 days.

For second preliminary study, SB was used instead of ET because SB has higher nitrogen content [9] and have higher moisture content than ET [19]. Nitrogen is the main nutrient for mycelium growth. The high nitrogen content will result the short time to complete the mycelium growth [20]. The mass of substrate was weighed to determine the performance of mycelium growth. The mass of substrates showed no changes after 10 days. Besides, the spawn did not showed any changes and no mycelium was produced.

During third preliminary study, only sugarcane bagasse substrate was used and inoculated with oyster mushroom spawn in order to measure the mycelium growth extension. The mycelium growth was measured as the mycelium spreading from the surface of substrate to the bottom. The mycelium extension in the SB was 0.28 cm/d. The study conducted by Hoa et al. [9] showed that, the SB has higher nitrogen (N), carbon (C) and C/N ratio which were 1.20%, 55.00% and 45.83, respectively compared to the sawdust. Naraian et al. [21] reported that the mycelium growth and primodial development of Pleurotus florida were dependent on the lignocellulosic materials, especially C/N ratio. Yang [22] also reported that high C/N ratio favored the mycelium growth and low C/N ratio favored fruiting body growth.

In summary, from the first preliminary experiment, EFB has the highest amount of mycelium. The experiment was stopped after 12 days since all the substrates took long time to fully colonize. During the second preliminary experiment, the experiment was unsuccessful because the oyster mushroom spawn used was ineffective, therefore no mycelium was produced after 10 days. Besides, the method to measure the mycelium growth in the substrates was not suitable. During the third preliminary experiment, the correct method to measure the mycelium growth and new oyster mushroom spawn was used. The result SB recorded 0.28 cm/d M. It showed a positive growth from SB. Therefore, EFB and SB were further used for factorial analysis.

3.2 Factorial analysis

Table 4 shows 16 runs of experiments were done for this study and the results of mycelium extension rate (M) and nitrogen concentration (N). The responses were analyzed using ANOVA by using Design Expert software V7, based on the value with 95% confidence level to identify the most contributing
factors and interaction between the factors on both responses M and N. M was ranged from 0.28 to 0.80 cm/d while N was ranged from 76 to 1040 mg/L. The lowest value of 0.28 cm/d of M was obtained using substrate B, 2.5 cm size of substrate, 1:14 SP/SS, incubated at ambient temperature and application of steam treatment. The highest value of 0.80 cm/d of M was obtained using substrate B, 2.5 cm size of substrate, 1:10 and 1:14 SP/SS, incubated at ambient temperature and no application of steam treatment. Meanwhile, the lowest value of 76 mg/L of N was obtained using substrate A, 2.5 cm size of substrate, 1:14 SP/SS, incubated at ambient temperature no application of steam treatment. Moreover, the highest value of 1040 mg/L of N was obtained using substrate B, 2.5 cm size of substrate, 25°C and application of steam treatment.

Table 4. Experimental results of mycelium extension rate (M) and nitrogen concentration (N).

| Run | A: Type of Substrate | B: Size of Substrates (cm) | C: SP/SS (g:g) | D: Temperature | E: Pretreatment of Substrate | Mycelium Extension Rate (M) (cm/d) | Nitrogen Conc. (N) (mg/L) |
|-----|----------------------|---------------------------|----------------|-----------------|------------------------------|-----------------------------------|--------------------------|
| 5   | A 0.5                | 1:10                      | 25°C           | Steam           | 0.60                         | 136                               |
| 8   | B 0.5                | 1:10                      | 25°C           | Non-Steam       | 0.53                         | 476                               |
| 9   | A 2.5                | 1:10                      | 25°C           | Non-Steam       | 0.79                         | 92                                |
| 14  | B 2.5                | 1:10                      | 25°C           | Steam           | 0.64                         | 1040                              |
| 3   | A 0.5                | 1:14                      | 25°C           | Non-Steam       | 0.79                         | 164                               |
| 13  | B 0.5                | 1:14                      | 25°C           | Steam           | 0.36                         | 560                               |
| 4   | A 2.5                | 1:14                      | 25°C           | Steam           | 0.54                         | 88                                |
| 2   | B 2.5                | 1:14                      | 25°C           | Non-Steam       | 0.79                         | 664                               |
| 15  | A 0.5                | 1:10                      | Ambient        | Non-Steam       | 0.78                         | 128                               |
| 7   | B 0.5                | 1:10                      | Ambient        | Steam           | 0.58                         | 356                               |
| 16  | A 2.5                | 1:10                      | Ambient        | Steam           | 0.32                         | 120                               |
| 11  | B 2.5                | 1:10                      | Ambient        | Non-Steam       | 0.80                         | 472                               |
| 6   | A 0.5                | 1:14                      | Ambient        | Steam           | 0.37                         | 80                                |
| 12  | B 0.5                | 1:14                      | Ambient        | Non-Steam       | 0.64                         | 572                               |
| 1   | A 2.5                | 1:14                      | Ambient        | Non-Steam       | 0.80                         | 76                                |
| 10  | B 2.5                | 1:14                      | Ambient        | Steam           | 0.28                         | 608                               |

3.3 Analysis of variance (ANOVA) for mycelium extension rate (M)

Table 5 shows the percentage contribution for each factor towards mycelium extension rate (M). The pre-treatment of substrate (E) has the highest percentage with value of 59.30%, followed by SP/SS (C) please put the value and temperature (D) please put the value, type of substrate (A) please put the value and lastly size of substrate (B) with value of 1.15%. ANOVA summary was shown in Table. 6 for mycelium extension rate (M) to estimate the coefficient of the model, to check the significance of each parameter and to indicate the interaction strength of each parameter. This model showed the coefficient of determination ($R^2$) was 0.8829. Olmez [23] suggest that a good fit of a bioprocess model, $R^2$ should be at least 0.80. Since the $R^2$ for this response variables is higher than 0.8, this model was accepted. It can be concluded that this model can be used for optimization.
Table 5. Contribution factor of mycelium growth extension rate (M).

| Factor                  | % Contribution |
|-------------------------|-----------------|
| A - Type of substrate   | 1.63            |
| B - Size of substrate   | 1.15            |
| C - SP/SS               | 2.63            |
| D - Temperature         | 2.63            |
| E - Pre treatment of substrate | 59.30        |

Table 6. ANOVA table for mycelium growth extension rate (M).

| Source                  | Sum of square | Df  | Mean Square | F value | P-value | Prob > F |
|-------------------------|---------------|-----|-------------|---------|---------|----------|
| Model                   | 0.46          | 9   | 0.051       | 5.02    | 0.0313  | Significant |
| A - Type of substrate   | 8.556E-03     | 1   | 8.556E-03   | 0.84    | 0.3957  |          |
| B - Size of substrate   | 6.006E-03     | 1   | 6.006E-03   | 0.59    | 0.4726  |          |
| C - SP/SS               | 0.014         | 1   | 0.014       | 1.35    | 0.2895  |          |
| D - Temperature         | 0.014         | 1   | 0.014       | 1.35    | 0.2895  |          |
| E - Pre treatment of substrate | 0.31      | 1   | 0.31        | 30.38   | 0.0015  |          |
| BD                      | 0.026         | 1   | 0.026       | 2.58    | 0.1593  |          |
| BE                      | 0.020         | 1   | 0.020       | 1.98    | 0.2085  |          |
| CE                      | 0.032         | 1   | 0.032       | 3.08    | 0.1298  |          |
| DE                      | 0.032         | 1   | 0.032       | 3.08    | 0.1298  |          |

3.4 Main effect and interaction effect between factors on mycelium extension rate (M)
The Pareto Chart in Figure 2 shows the main effects and interaction effects of the factors for M. For the main effect, it showed that there are three main factors contribute to M. Pre treatment of substrate (E) has shown the highest effects followed by SP/SS (C) and temperature (D). In Table 5, Factor E gave the highest contribution among other factors. Based on Table 1, for Factor E, steam was set as low level and non steam was set as high level. Meanwhile Factor C, 1:10 was set as low level and 1:14 was set as high level. Negative effect is when the factor is not proportional to the response value. From Figure 2, Factor E and Factor C gave negative effect towards the M. Therefore, when the Factor E and Factor C is at low level, the value of M is increasing. On the other hand, Factor D gave positive effect towards the M. Positive effects is when the factor is proportional to the response value. Therefore, when Factor D is increasing, the value of M is increasing as well. For interaction effects, it showed there were two interaction effects that contributed in M which were SP/SS and pre treatment of substrate (CE) and temperature and pre treatment of substrate (DE) with negative and positive effects, respectively.
3.5 Effect of independent processing parameters on mycelium extension rate (M)
The effect of three independent variables on M which were SP/SS, temperature and pre treatment of substrate is shown in Figure 3a, Figure 3b and Figure 3c respectively. From Figure 3a, there was no significant difference of SP/SS on M because at ratio 1:14, M achieved 0.81 cm/d while at ratio 1:10, M was 0.78 cm/d. This was supported by the information from Pareto Chart (Figure 2) where SP/SS factor was below than t-value limit. Banala et al. [24] reported that the coefficients with t-value of effect above Bonferroni line are designated as certainly significant coefficients, and coefficients with t-value of the effect below the t-limit line is statistically insignificant to the response. From Figure 3b, at ambient temperature (28 to 30°C), M was 0.81 cm/d but at 25°C, the value of M was 0.73 cm/d which was lower than ambient temperature. It showed that there was no significant difference between both temperatures. This is because the range of temperature used in this study was in the range of optimum temperature (25 to 30°C) for mycelium growth of oyster mushroom Pleurotus spp. [6]. This finding was similar with the studies of Hoa and Wang [25] whereas the optimum temperature for Pleurotus species was in the range of 24°C to 32°C (ambient). From Figure 3c, M was 0.61 cm/d when there was no treatment applied and the value of M become higher which was 0.81 cm/d when steam treatment was applied. This is because the steam pasteurization can reduce the amount of microscopic competitors in substrates. This gives the mycelium an advantage over harmful organisms, allowing it to take over the substrate and eventually produce the mushrooms [26].
(a) SP/SS

(b) Temperature
3.6 Interaction effects between factors on mycelium extension rate (M)

Figure 4a shows the interaction effect between SP/SS and pre treatment of substrate (CE) on M. When steam treatment (red line) was applied, it gave better performance on M. There was no significant effect of SP/SS on M because at ratio 1:14, M was 0.81 cm/d. Meanwhile at ratio 1:10, M achieved was 0.78 cm/d. When no treatment (green line) was applied, SP/SS showed significant effect on M. As indicated by Pareto Chart (Figure 2), the most significant factor was pretreatment because it was above Bonferroni Limit. The effect of pretreatment is the most significant when no treatment was applied. At ratio 1:10, M achieved 0.60 cm/d and the value become lower which was 0.46 cm/d at ratio 1:14. According to Beyer [28], by pasteurizing the substrate with steam can optimize the condition of the substrate by reducing or eliminating the bad microorganism and prevent competition between the spawn and other microorganism. When there was no application of steam to substrate, spawn needs to compete for the substrate with other microorganism. Thus, more spawn will give better performance of M [29]. In this study, the amount of spawn at ratio 1:10 was more than at ratio 1:14, therefore M achieved better performance at ratio 1:10 when no pretreatment applied. Figure 4b shows the interaction effect between temperature and pre treatment of substrate (DE) on M. It was the same as interaction effect of CE where M recorded higher value when steam treatment (red line) was applied. When steam treatment was applied, there was no significant difference of temperature on M because M achieved 0.81 cm/d at ambient temperature (28 to 30°C) and 0.78 cm/d at 25°C. Meanwhile, when no treatment (green line) applied, M was higher at ambient temperature because the optimum temperature for Pleurotus sp. is between 28 to 30°C [25].
Figure 4. Analysis of interaction effects on mycelium extension rate ($M$) (a) Factor SP/SS and pre-treatment ($CE$); and (b) Factor of temperature and pre-treatment ($DE$).

### 3.7 Analysis of variance (ANOVA) for nitrogen concentration in mycelium ($N$)

Table 7 shows the percentage contribution for each factor towards nitrogen concentration in mycelium ($N$). The type of substrate ($A$) has the highest percentage with value of 75.80%, followed by temperature ($D$) at 3.31%, size of substrate ($B$) at 2.40%, pretreatment of substrate ($E$) at 0.60% and lastly SP/SS has the least percentage with value 0.0003%. ANOVA summary was shown in Table 8. This model showed $R^2$ was 0.8829. Since the $R^2$ for this response variables is higher than 0.8, this model was accepted. It can be concluded that this model can be used for optimization.
Table 7. Contribution factors of nitrogen concentration in mycelium (N).

| Factor                        | % Contribution |
|-------------------------------|----------------|
| A - Type of substrate        | 75.80          |
| B - Size of substrate        | 2.40           |
| C - SP/SS                     | 0.0003249      |
| D - Temperature               | 3.31           |
| E - Pre treatment of substrate| 0.60           |

Table 8. ANOVA table for nitrogen concentration in mycelium (N).

| ANOVA for selected factorial model | Source          | Sum of square | Df | Mean Square | F value | P-value Prob > F |
|-----------------------------------|-----------------|---------------|----|-------------|---------|-----------------|
| Model                             | 1.209E+06       | 12            |    | 1.007E+05   | 13.59   | 0.0270 Significant |
| A - Type of substrate             | 9.332E+05       | 1             | 9.332E+05 | 125.85     | 0.0015 |
| B - Size of substrate             | 29584.00        | 1             | 29584.00  | 3.99       | 0.1397 |
| C - SP/SS                         | 4.00            | 1             | 4.00      | 2.395E-04  | 0.9829 |
| D - Temperature                   | 40804.00        | 1             | 40804.00  | 5.50       | 0.1007 |
| E - Pre treatment of substrate    | 7396.00         | 1             | 7396.00   | 1.00       | 0.3915 |
| AB                                | 56644.00        | 1             | 56644.00  | 7.64       | 0.0699 |
| AD                                | 26896.00        | 1             | 26896.00  | 3.63       | 0.1529 |
| BC                                | 20164.00        | 1             | 20164.00  | 2.72       | 0.1977 |
| BE                                | 36100.00        | 1             | 36100.00  | 4.87       | 0.1145 |
| CD                                | 17424.00        | 1             | 17424.00  | 2.35       | 0.2228 |
| CE                                | 24336.00        | 1             | 24336.00  | 3.28       | 0.1677 |
| DE                                | 16384.00        | 1             | 16384.00  | 2.21       | 0.2339 |

3.8 Main effect and interaction effect between factors on nitrogen concentration in mycelium (N)

The Pareto Chart in Figure 5 shows the main effects and interaction effects of the factors to N. For the main effect, it shows that type of substrate (A) is the highest main contributing factors to N. In Table 7, Factor A gave the highest contribution among other factors. For Factor A (Table 1), SB was set as low level and EFB was set as high level. Positive effect is when the factor is proportional to the response value. From Figure 4, Factor A gave positive effect towards the N. Therefore, when the Factor A is at high level (EFB), the value of N is increasing. For interaction effects, type of substrate and size of substrate (AB) gave the highest positive contribution to N.
3.9 Effect of independent processing parameters on nitrogen concentration in mycelium (N)

Figure 6 shows the effect of two independent variables (type of substrate and temperature) on N. As supported by Pareto Chart (Figure 5), the type of substrate was the most significant factor to N because it was above Bonferroni Limit. N achieved 656 mg/L when substrate B (EFB) was used, however the value of N become lower (27.5 mg/L) when substrate A (SB) was used (Figure 6a). According to Widiastuti and Tri-Panji [30] and Hoa et al. [9], the nitrogen content in EFB and SB was 2.34% and 1.20%, respectively. Ortega et al. [7] described a nitrogen increase in mushroom mycelium related to the amount of nitrogen in the initial substrate. Therefore, nitrogen content in substrate is proportional to N. Figure 6b shows that studied temperatures (between 25°C to ambient temperature) did not give significant effect to N. N was 656 mg/L at ambient temperature while it become just a little bit lower (603 mg/L) at 25°C.
Figure 6. Analysis of most effective independent factors in nitrogen concentration ($N$) (a) Type of substrate; and (b) Temperature.

3.10 Interaction effects between factors on nitrogen concentration in mycelium ($N$)
Figure 7 shows the interaction effect between the type of substrate and size of substrate (AB) on N. When using substrate B, for both 2.5 cm (red line) and 0.5 cm (black line) size of substrate, the N value achieved 664 mg/L and 617 mg/L, respectively which was higher than substrate A. Besides, there was no significant difference of size of substrate on N when using substrate B but it showed the significant difference when using substrate A. As indicated by Pareto Chart (Figure 4), the most
significant factor was type of substrate because it was above Bonferroni Limit while size of substrate was insignificant factor to N since it was below than t-limit. Therefore, the type of substrate has important effect on N value. N achieved was 171 mg/L at 0.5 cm and become lower which was 27 mg/L at 2.5 cm size of substrate. According to Bellettini et al. [11], the substrate that has low nitrogen content in the range of 0.03% to 1.5% can be cut into small size to increase the surface area of the substrate and nitrogen content in the substrate. The substrate A (SB) has 1.20% nitrogen while substrate B (EFB) has 2.34% nitrogen, so substrate A has lower nitrogen content than substrate B [29, 20]. Thus for substrate A, by cutting the substrate into 0.5 cm, it gave better performance on N than 2.5 cm.

![Figure 7. Interaction factors of type of substrate and size of substrate (AB)](image)

### 3.1.1 Validation of experiment

Validation experiment was conducted based on the suggested of the best conditions by Design Expert Software (Table 9). Three runs were conducted in order to compare the predicted result from Design Expert software and the actual result. The error was calculated by using Eq. (1). Table 10 shows the comparison of predicted and actual data of M and N. Run 1 was selected due to the lowest value of error compared to others. Thus, the actual results from suggested of the best condition were 0.8 cm/d of M and 656 mg/L of N.

$$\text{Error calculation} = \frac{|(\text{actual} - \text{predict})|}{\text{actual}} \times 100$$  \hfill (1)

### Table 9. Suggested best condition for Pleurotus sp. Cultivation.

| Item                        | Details       |
|-----------------------------|---------------|
| Type of substrate           | B (EFB)       |
| Size of substrate           | 2.5 cm        |
| SP/SS                       | 1:14          |
| Temperature                 | Ambient       |
| Pre treatment of substrate  | Steam         |
Table 10. Results from suggested best condition.

|                  | Mycelium extension rate ($M$) (cm/d) | Nitrogen concentration ($N$) (mg/L) |
|------------------|-------------------------------------|-------------------------------------|
|                  | Predicted  | Actual  | Error (%) | Predicted  | Actual  | Error (%) |
| Run 1            | 0.8125     | 0.8     | 1.5625    | 656.5      | 656     | 0.076     |
| Run 2            | 0.8125     | 0.8     | 1.5625    | 656.5      | 645.2   | 1.751     |
| Run 3            | 0.8125     | 0.8     | 1.5625    | 656.5      | 664     | 1.130     |

4. Conclusion
The purpose of this study is to evaluate the factors affecting the mycelium growth in *Pleurotus* sp. cultivation by using agricultural waste. The results showed that the most contributing factor for mycelium extension rate ($M$) was pre treatment of substrate while the type of substrate was the most significant factor for the nitrogen concentration ($N$). The best conditions for the mycelium growth of *Pleurotus* sp. cultivation were using substrate B (EFB), 2.5 cm size of substrate, 1:14 SP/SS, incubated at ambient temperature and application of steam treatment to substrate with 0.8 cm/d of $M$ and 656 mg/L of $N$. It can be concluded that the agricultural waste such as empty palm fruit bunch (EFB) can replace the sawdust as a media for oyster mushroom cultivation.

Acknowledgement
The author wishes to acknowledge the Universiti Malaysia Pahang for funding the project under grant RDU1803119.

Conflict of interest statement
The authors have NO affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Symbols
A  Type of substrate
ANOVA Analysis of variance
B  [cm] Size of substrate
BS Banana stem
C  [g/g] SP/SS
CF Coconut fiber
CG Coffee ground
D  [$^\circ$C] Temperature
E  Pre treatment of substrate
EFB Empty palm fruit bunch
ET Egg trays
M  [cm/d] Mycelium extension rate
N  [mg/L] Nitrogen concentration in mycelium
SB Sugarcane bagasse
SP/SS [g/g] Mass ratio of spawn to substrate
TLFA Two level factorial analysis

References
[1] Kong WS 2004 *Mushroom Growers’ Handbook 1* (Seoul: MushWorld)
[2] Pathmashini L, Arulnandhy V and Wijeratnam R W 2009 *Ceylon Journal of Science (Biological Sciences)* 37 (2) 177-182
[3] Bernandri E, Minotto E and Nascimento J S D 2013 *Arquivos do Instituto Biologico* 80(3) 318-324
[4] Madan M L, Vasudevan P and Sharma S 1987 *Biological Wastes* 22(4) 241-250
[5] Miles P G 1997 *Mushroom Biology: Concise Basics and Current Developments 1* (Singapore: World Scientific Publishing)

[6] Neelam S, Chennupati S and Singh S 2013 *Asian J. Plant Sci. Res.* 3(1) 163-169

[7] Ortega M, Martinez E and Betancourt D 1992 *World J. Microb. Biot.* 8(4) 402-405

[8] https://www.pressreader.com/malaysia/the-star-malaysia/20170221/281621010100603 (Accessed December 7, 2017)

[9] Hoa H T, Wang C L and Wang C H 2015 *Microbiology* 43(4) 423-434

[10] Barshteyn V and Krupodorova T 2016 *J. Microbiol. Biotechn. Food Sciences* 5(6) 563-577

[11] Bellettini M B, Fioda F A and Maieves H A 2016 *Saudi J. Biol. Sci.* 1-14

[12] Biswas S, Datta M and Ngachan S 2011 *Mushrooms: A Manual for Cultivation 2* (New Delhi: PHI Learning)

[13] http://www.usahawan.com/idea-bisnes/projek-tanaman-cendawan.html/ (Accessed December 5, 2017)

[14] Fan L and Carlos S 2005 *Mushroom Growers Handbook 2* (Seoul: Mush World)

[15] https://www.statease.com/pubs/whats_new_in_DX9.pdf (Accessed on March 3, 2018)

[16] Wahab R, Mohd Dom S M and Mustafa M T 2015 *Plant Sci. J.* 10(5) 179-190

[17] Chang X and Miles P 2004 *Mushrooms: Cultivation, Nutritional Value, Medicinal Effect and Environmental Impact 2* (New York: CRC Press)

[18] Pandey A, Soccol C and Mitchell D 2000 *Process Biochemical* 35(10) 1153-1169

[19] Hamsalu Mosisa Z, Kebede A and Preetha V 2015 *Int. J. Adv. Res.* 3 (2) 522-531

[20] http://eprints.utm.my/id/eprint/8680/ (Accessed on July 18, 2018)

[21] Naraian R, Sahu S and Kumar S 2009 *The Environmentalist 29*(1) 1-7

[22] Yang X 2000 *Cultivation of edible mushroom* (Beijing: China Agricultural Press)

[23] Olmez T 2009 *J. Hazard. Mater.* 162 (2-3) 1371-1378

[24] Banala V T, Srinivasan B and Rajamanickam D 2013 *ISRN Pharmaceutics* 1-15

[25] Hoa H T and Wang C L 2015 *Mycobiology* 43(4) 423-434

[26] http://www.mushroom-appreciation.com/pasteurize.html#sthash.M7taJPX0.dpbs (Accessed on July 7, 2017)

[27] Oseni T O, Dlamini S O and Earnshaw D M 2012 *Int. J. Agric. Biol.* 14(2) 251-255

[28] https://extension.psu.edu/growing-mushrooms-microbial-activity-in-substrate (Accessed on August 18, 2017)

[29] http://urn.kb.se/resolve?urn=urn:nbn:se:slu:epsilon-s-4236 (Accessed on December 21, 2017)

[30] Widiastuti S and Tri-Panji A 2015 *Biopropal Industry* 6(2) 73-80

https://media.neliti.com/media/publications/54525/doi:10.5897/AJAR11.2350

[31] Cheng Z, Wu Q and Huang J B 2013 *Afr. J. Agric. Research* 8(33) 4390-4395 DOI: 10.5897/AJAR11.2350

[32] Cho S B 2004 *Oyster Mushroom Cultivation 1* (Seoul: MushWorld)

[33] Chanakya H 2015 *Energy for Sustainable Development* 27 84-92

[34] http://www.disknet.com/indiana_biolab/b062.htm (Accessed on March 13, 2018)

[35] Zhang R, Li X and Fadel J 2002 *Bioresource Technology* 82(3) 277-284

[36] Bhatti M, Jiskani M and Wagan K 2007 *Pak. J. Bot.* 39(7) 2685-2692

[37] Ali M A, Hussain S and Nawaz R 2004 *J. Agric. Residues* 42(2) 201-209