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# Preliminary study on *Azolla* cultivation and characterization for sustainable biomass source

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**Abstract**. *Azolla* is a freshwater fern that belongs to the *Azollaceae* family. It is easy to grow and is highly productive. It can fix atmospheric nitrogen due to the presence of *Anabaena azolla*e. *Azolla* has been applied to the rice field as a classic fertilizer. It is a good source of protein and contains almost all essential amino acids and minerals. Various research has been done and is still ongoing to determine the capability of *Azolla* as a phytoremediator and to be used as a sustainable bioenergy source. This preliminary study investigated the ideal environment for *Azolla* cultivation in Malaysia (humid weather throughout the year with average daily temperature across Malaysia between 21°C and 32°C). To the best of our knowledge, there is no research conducted in Malaysia to study the optimum environment for *Azolla* cultivation. Therefore, determining the optimum condition for growing *Azolla* was done by manipulating parameters: water depth, nutrient concentration, pH, and sunlight exposure. Meanwhile, chemical compositions (moisture, crude protein, crude fat, ash, crude fibre, carbohydrate and energy) were determined using proximate analysis. Results obtained showed that *Azolla* growth was the best in water depth of 20 cm, the nutrient concentration of 812.5 ppm, pH of 7 and under 100% sunlight exposure. Dried *Azolla* had 6.38% moisture, 27.1% crude protein, 6.37% crude fat, 14.29% ash, 34.29% crude fibre, 45.86% carbohydrate and 349.17 kcal/100 g energy. Based on the result, *Azolla* cultivated in this experiment could be used as a sustainable biomass source to produce animal feed (high protein content) and bioenergy (high fibre content).

## 1. Introduction

*Azolla* is a freshwater fern that belongs to the *Azollaceae* family. It is easy to grow and can be grown on any open tank [1]. *Azolla* is highly productive and can double its biomass in 5.6 days [2]. It can fix atmospheric nitrogen because of *Anabaena azollae* (blue-green algae) in the lobes of *Azolla* leaves [3]. As for that, *Azolla* is applied to the rice field as a classic fertilizer [4, 5]. *Azolla* is also a good protein source and contains almost all essential amino acids and minerals like iron, calcium, magnesium, potassium, phosphorus, and manganese [6]. *Azolla* is also used as feed due to its high protein and amino acid content compared to soybean [7]. Various research has been done and is still ongoing to utilize *Azolla* as a phytoremediator [8] and sustainable bioenergy source [9-13].

As a phytoremediator, *Azolla* was able to remove 63.0% chemical oxygen demand (COD), 70.5% biological oxygen demand (BOD), 51.0% ammonia, 65.4% phosphate and 53.8% nitrates from palm oil mill effluent (POME) [14]. Sequential treatment of wastewater containing high levels of phosphate by *L. punctata* followed by *Azolla* led to complete removal of $NH_4$, $NO_3$ and up to 93% reduction of $PO_4$ [12]. Meanwhile, as a feedstock for livestock and bioenergy, *Azolla* contain an appropriate amount of protein, lipid and fibre which is comparable to the existing sustainable source. Therefore, this research was intended to determine optimum growing condition for *Azolla* together with its nutritional analysis. The result for *Azolla* nutritional analysis would determine whether *Azolla* grown in optimum condition suit to be feedstock for agricultural sector or bioenergy sector.

## 2. Methodology

The experiment involved two main steps i.e., the cultivation of *Azolla* and the determination of nutrient content of dried *Azolla* grown under optimum growing conditions using proximate analysis.

### *2.1. Cultivation of Azolla*

Sixteen rectangular open-top aquaculture tanks (80 cm x 50 cm x 25 cm) were built at Agro-site UTM, JB to grow *Azolla*. All the tanks were exposed to direct sunlight. The tanks were covered with the greenhouse plastic film to prevent the raining water from reaching *Azolla* and water medium as it could change the properties of water and thus affect the growth of *Azolla*. *Azolla* was cultivated using different parameters (water depth, nutrient concentration, pH, and sunlight exposure) to determine its optimum growing condition. The optimum growth for *Azolla* was obtained by measuring its biomass and was represented by its growth value. After growing *Azolla* for six days, its biomass was measured and the growth value was determined. Growth value (GV) is the ratio of final weight to the initial weight of *Azolla* under a certain period of observation. (GV= wet weight of harvested plants/wet weight of initial explants).

*2.1.1. Manipulating water depth.* Water pH was fixed at 7 using tap water, 85% ortho-phosphoric acid and Ocean Free Super $GH^+$. Rabbit manure compost was used to make 437.5 ppm nutrient concentration in all tanks. Different amounts of compost were used based on different water depths as shown in Table 1. 25 g of fresh *Azolla* was cultivated in four aquaculture tanks with a water depth of 5 cm, 10 cm, 15 cm, and 20 cm.

**Table 1.** Amount of compost used based on different water depths.

| Water depth(cm) | Compost (g) |
|---|---|
| 5 | 8.75 |
| 10 | 17.50 |
| 15 | 26.25 |
| 20 | 35.00 |

*2.1.2. Manipulating nutrient concentration.* The water pH was fixed at 7 and water depth was set at 20 cm. 25 g of fresh *Azolla* was cultivated in four aquaculture tanks with water. Each tank was supplied with a different weight of compost. Four different weights of compost (35 g, 65 g, 130 g and 260 g) were added to the tanks. These indicated the concentration of 437.5, 812.5, 1625.0 and 3250.0 ppm respectively.

*2.1.3. Manipulating pH.* The water depth was fixed at 20 cm, and compost was added to the water by 65 g (812.5 ppm). 25 g of fresh *Azolla* was cultivated in four aquaculture tanks with water pH set at 3, 5, 7 and 7.5. The water with pH 3 and 5 was adjusted using 85% of ortho-phosphoric acid. Meanwhile, the water with pH 7.5 was adjusted using Ocean Free Super $GH^+$ (solution for a slight pH increase). The tap water was used as water at pH 7. The pH meter was used to measure the pH of the water while adjusting the pH.

*2.1.4. Manipulating light exposure.* 25 g of fresh *Azolla* was cultivated in four aquaculture tanks with all water pH set at 7 and compost of 65 g (812.5 ppm) was added to the water. The water depth for all sets of the experiment was fixed at 20 cm. One of the tanks was left without the cover of the orchid shade net. The rest of the three tanks was covered with the orchid shade net with 10%, 30% and 70% light penetration respectively.

### *2.2. Proximate Analysis of Azolla*

The *Azolla* were grown under optimum conditions and harvested after six days. They were dried in the oven at 105°C for 4 h and ground into powder. The dried sample was used in the proximate analysis for its moisture, crude protein, crude fat, ash, crude fibre, carbohydrate, energy and total sugar content. Table 2 below describe experimental procedures for the estimation of each nutrient.

**Table 2.** Experimental procedures for proximate analysis [15].

| Analysis (content) | Experimental Procedure |
|---|---|
| Ash | 2 g of the sample was weighed ($W_1$) in a crucible and heated in a furnace at 550°C for 6 h until light gray ash residue or to constant weight. It was cool in a desiccator and weighed ($W_2$). The % ash was determined using Equation (1): $$\% \text{ ash} = W_2 / W_1 \times 100 \quad (1)$$ |
| Moisture | A dish was heated, cool and weighed ($W_1$). 2 g ($W_2$) of the sample was placed into the dish. The dish was oven-dried for 5 h at 95 – 100°C. The sample was transferred to a desiccator and weighed soon after reaching room temperature ($W_3$). The % moisture was determined using Equation (2): $$\% \text{ moisture} = W_2 - (W_3 - W_1) / W_2 \times 100 \quad (2)$$ |
| Crude fat | Crude fat content was determined by the Soxhlet extraction method. 2 g of moisture-free sample was weighed (W1). The dried extraction flask was weighed (W2). The sample was extracted with petroleum ether at 80°C for 8 h. The extraction flask was removed from the extraction apparatus and the solvent collected into the extraction flask was cautiously distilled on the water bath. The flask was dried at 100°C (1 hour) in the drying oven, removed from the oven and cooled in a desiccator for at least 1 hour before being weighed (W3). The % fat was determined using Equation (3): $$\% \text{ crude fat} = (W_3 - W_2) / W_1 \times 100 \quad (3)$$ |
| Crude protein | The crude protein was determined using the Kjeldahl method. The conversion factor of 6.25 was used to calculate crude protein by multiplying it with evaluated nitrogen. |
| Crude fibre | 2 g (Ws) of the sample was treated with 200 ml 1.25% H2SO4 and boil for 30 minutes. The sample was filtered and washed with hot water before being treated and boiled with 200 ml 1.25 % NaOH for 30 minutes. The sample was filtered again and washed with hot water. The residue was transferred into a crucible and oven-dried for 2h at 230°C. The crucible was cool inside the desiccator and weighed after 20 minutes (W1) to get the weight of the crucible with fibre. The crucible was then placed inside the furnace at 550°C for 2 h. The crucible was let cooled inside the desiccator and weighed again after 20 minutes (W2) to get the weight of the crucible with ash. The % of crude fibre was determined using Equation (4): $$\% \text{ crude fibre} = (W_2 - W_1) / W_s \times 100 \quad (4)$$ |
| Carbohydrate | The percentage of carbohydrate was calculated using the equation (5): $$\% \text{ carbohydrate} = 100 - (\% \text{ ash} + \% \text{ moisture} + \% \text{ crude fat} + \% \text{ crude protein}) \quad (5)$$ |
| Energy Level | To calculate the energy available from a food, multiply the number of grams of carbohydrate, protein, and fat by 4, 4, and 9, respectively. Then add the results together. |

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## 3. Result and Discussion

### 3.1. Cultivation of *Azolla*

The optimum condition of growing *Azolla* using different parameters (water depth, nutrient concentration, pH and sunlight exposure) are shown in Figure 1 below.

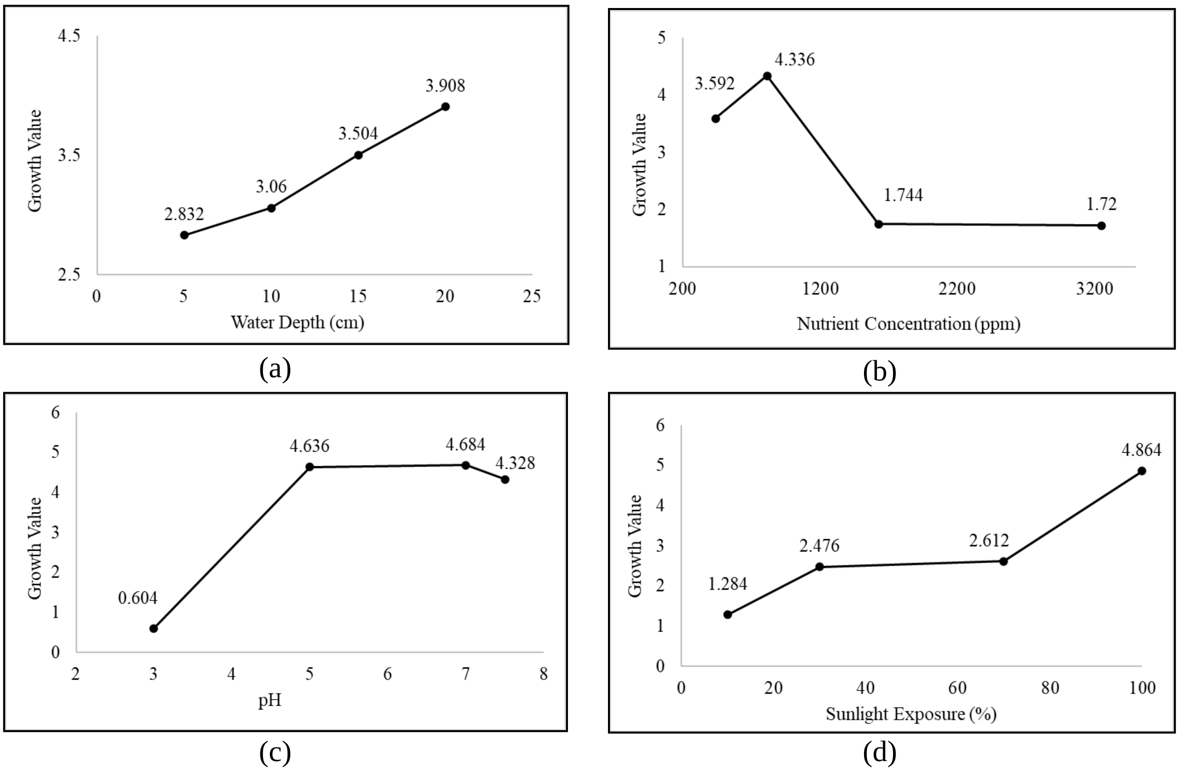


**Figure 1.** Growth value of *Azolla* against (a) water depth (cm), (b) nutrient concentration (ppm), (c) pH and (d) sunlight exposure (%).

Figure 1 (a) showed that the growth value of *Azolla* is proportional to the water depth. Water depth of 20 cm showed the highest growth value (3.908) of *Azolla* compared to water depth of 5 cm (2.832). The result is similar to research conducted by Biswas [16] which also showing that *Azolla* growth favourably in deeper water depth. Likewise, other research conducted showing water depth affects the growth value of *Azolla* [17, 18]. The best explanation might be due to the plants' ability to obtain sufficient dissolved oxygen (DO) in water for growth. Although aquatic plants depend on carbon dioxide for photosynthesis, they also rely on aerobic respiration to produce energy for growth, maintenance and active uptake of ions by roots [19]. Roots do a lot of 'heavy lifting' in a plant, literally, they are pumping ions across membranes to pull in water, to concentrate other nutrients that the plant needs for survival and growth, and to pressurize the plant enough for those nutrients to make it up into the leaves. Those processes take energy, and in turn they need oxygen [20]. For roots of *Azolla*, it is fully submerged in the water. Thus, depletion of DO can affect the growth of *Azolla*. The higher the water depth in a tank, the bigger the volume of water. Theoretically, higher volume of water could hold more DO than lower volume of water. Hence, providing the roots of *Azolla* with sufficient DO. Moreover, water at deeper level is colder compared to at the surface. Water at the surface is exposed to direct sunlight making it warmer than water at deeper level. Cold water hold more DO [21] because the water molecules are closely packed together making it difficult to release into the atmosphere. Besides, solubility of oxygen is decreased with warm water [22] making it holding lesser DO.

Figure 1 (b) showed the growth value of *Azolla* against nutrient concentration. Optimum growth of *Azolla* needs 5 mg/L (5 ppm) of phosphorus [23]. Water without phosphate showed low growth [24] and biomass [25] of *Azolla.* The rabbit manure compost normally contains 0.5-1% of phosphorus [26]. Thus, the concentration of rabbit manure compost (nutrient concentration) in water should be between 500-1000 mg/L to obtained phosphorus content of 0.5-1%. The highest growth value of *Azolla* obtained in this research was when the nutrient concentration is at 812.5 ppm. The growth value of *Azolla* increased when the concentration of nutrient increases up to a certain extent as can be seen in this research. Doubling compost concentration resulted in drastic reduction in the growth value of *Azolla.* It is because excessive usage of the amount of compost caused higher water turbidity and in turn lowering the DO level of the water. Turbidity is caused by particles of soil and organic matter in the compost suspended in the water tank. These particles scatter light and make the water appear cloudy or murky. Turbidity affects the growth rate of *Azolla* because increased turbidity causes a decrease in the amount of light for photosynthesis. Turbidity can also increase water temperature because suspended particles absorb more heat [27], leading to a decrease in DO [28]. Besides, more compost means more organic matters as well as microbes. Microbes used DO to decompose organic material [29] and in that manner, competing with *Azolla* roots to get DO. Under oxygen-deficient conditions, the root cell energy pool is greatly decreased. This energy is necessary for *Azolla* uptake of mineral nutrients by active processes. Hence, phosphate and potassium uptake are decreased and affect the growth of *Azolla* [30].

Figure 1 (c) revealed a consistent result with the trend stated in a review by Sadeghi whereby *Azolla* can survive within a water pH range of 3.5 to 10, but optimum growth occurs in the pH range of 4.5 to 7 [31]. In this research, *Azolla* showed highest growth value when the pH of water was 7. There was also not much difference in growth value for that of pH 5 and 7.5. However, when it came to pH 3, its growth value was below 1. pH 1 was too acidic and corrosive to the plant. *Azolla* exhibit retarded growth and changes in the leaves colour from green into yellow or brown. Acidic pH led to $H^+$ toxicity and damage the roots. Research conducted to citrus showed the roots were damaged when exposed to pH 2.5, affecting the uptake of minerals, nutrients, and water [32]. This inhibited the growth of plants. Low pH also increases the solubility of minerals such as aluminium and causing the water medium to contain toxic level of aluminium [33]. Plant growth can be repressed in the medium with severely high aluminium levels due to photosynthetic inhibition [34]. Therefore, exposure to acid water does not directly kill it, but the effects of low pH conditions (leaf damage, nutrient deficiencies and mineral toxicity) are detrimental to the plants.

Figure 1 (d) revealed that growth value of *Azolla* is proportional to the percentage of sunlight exposure. The one with 100% sunlight exposure exhibited the highest growth, as seen in another research. It is recommended to grow *Azolla* in full sun shine [35] and direct sunlight [36]. Increased photosynthesis in 100% light intensity led to production of high fresh matter whereas reduced light intensity decreased photosynthesis, producing low fresh matter. In conformity to this, under shade *Azolla* have the lowest fresh matter production. Nevertheless, research conducted by [37] showing that light intensity of 70% is the best for growing *Azolla*. The difference found might be due to the usage of different species of *Azolla* and using different water sources (brackish instead of tap water).

### *3.2. Proximate Analysis of Azolla*

The result for proximate analysis of *Azolla* is shown in Table 3 below.

**Table 3.** Proximate Analysis of dried *Azolla*

| Composition | Value | Composition | Value |
|---|---|---|---|
| Moisture (%) | 6.38 | Crude Fat (%) | 6.37 |
| Ash (%) | 14.29 | Crude Fibre (%) | 34.29 |
| Crude Protein (%) | 27.10 | Carbohydrate (%) | 45.86 |
| | | Energy (kcal/100g) | 349.17 |

Table 3 showed that the moisture content of *Azolla* in this study is 6.38% which is lower than the study conducted by other research [38-40] which showing moisture content to as high as 94% [38]. The differences might be due to the duration of drying, temperature used to dry the samples and type of sample used to determine the moisture content (dried sample/wet sample). Sample used in this experiment was dried *Azolla.* Ash was found to be at the value of 14.29%. Other research showing the *Azolla* either with a lower percentage of ash (2 –12.5%) [38, 39, 41] or higher percentage of ash content (15.5 – 25.5%) [42-45]. Meanwhile, the crude protein of *Azolla* is 27.10% and the value is higher than other studies conducted, showing the percentage value of between 18 to 27% [38, 40-45]. The crude protein content also can be as high as 35% [46]. High protein content of *Azolla* suggests that it's a potential natural protein source. Hence, it has been used as a replacement for soybean meal [47], fodder mixture [38] or to feed livestock [41, 43-46, 48-50].

The percentage of crude fat is 6.37% and the value is higher than the study conducted in these research [38, 39, 41, 43-45], which shows the range of crude fat between 0.5% - 4.5%. Further test could be conducted to determined types of fatty acids present in the sample because research by [9, 10, 51] showed that *Azolla* contains fatty acids with good biodiesel property and meets most of the important requirement for biodiesel standards. *Azolla* also was investigated to be produced as bio-oil [40]. Pyrolysis of *Azolla* showed that this plant could be used as feedstock for production of bio-oil and biodiesel [8]. Table 4 below summarize the comparison of proximate analysis value of *Azolla* in this research and other research.

**Table 4.** Proximate Analysis of *Azolla* in this research and other research

| Composition | This study | Lower value | Higher value |
|---|---|---|---|
| Moisture (%) | 6.38 | - | 94.68 [38]*<br>91.77 - 92.25 [39]*<br>10.9 [40] |
| Ash (%) | 14.29 | 12.51 [38]<br>2 [39]<br>10.5 [41] | 20.3±0.28 [42]<br>15.53 [43]<br>17.34 [44]<br>25.5 [45] |
| Crude Protein (%) | 27.10 | 24.51 [38]<br>18.34 [40]<br>24-30 [41]<br>26.5±0.08 [42]<br>20.2 [43]<br>22.48 [44]<br>22.25 [45] | 25 - 35 [46] |
| Crude Fat (%) | 6.37 | 4.23 [38]<br>0.6 - 1.8 [39]<br>3-3.36 [41]<br>3.53 [43]<br>4.5 [44]<br>2.45 [45] | - |
| Crude Fibre (%) | 34.29 | 11.77 [38]<br>12.12 [43]<br>14.7 [44]<br>11.19 [45] | - |

* Wet sample

Crude fibre content of *Azolla* in this research is higher (34.29%) than in another research (11 – 15%) [38, 43-45]. The main components of fibre are cellulose, hemicelluloses, lignin, pectin, and wax [52] which can be treated to produce sugar. Hence, plants with high fibre contents are extensively studied to produce bioethanol and biohydrogen. For example, cellulose in rice husk and rice bran was treated with exoenzyme cellulase to produce fermentable sugar that can be used to produce bioethanol [53]. Similarly, lignocellulose substrate in *Azolla* undergoes biological pretreatment to produce sugar for bioethanol production [54-57]. *Azolla* also undergo enzymatic hydrolysis to produce sugar to be used as a substrate in biohydrogen production [11, 12, 58, 59]. Carbohydrate content of *Azolla* is high 45.86%

and this indicated that *Azolla* has high fibre content. As stated before, high fibre content makes *Azolla* an attractive plant to be used as sustainable biomass source for bioenergy.

## 4. Conclusion

*Azolla* is a macrophyte which can be easily grown in Malaysia climate. Its optimum growing conditions was found to be in water depth of 20 cm, nutrient concentration of 812.5 ppm, pH level of 7.0 and exposure to 100% sunlight. *Azolla* grown in optimum condition has high percentage content of fat, protein and fibre which could be used as a sustainable biomass source for livestock feed and bioenergy.

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