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The Influence of Light Intensity on the Protein Content of *Azolla Microphylla* and Pre-treatment with *Saccharomyces cerevisiae* to Increase Protein Recovery

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ABSTRACT

This study was carried out to examine the effect of light intensity to the growth and protein content of *Azolla microphylla*. The light intensity was varied from 10-465 W/m^2 using three different type of shading materials; vinyl, polyethylene and 50% shade net. The protein content of *Azolla microphylla* was extracted at different pH (2-12) and pre-treatment with *Saccharomyces cerevisiae*. The highest relative growth rate of 0.18 g.g^{-1}d^{-1} was obtained when *Azolla microphylla* was cultivated using a transparent vinyl plastic with an average maximum light intensity of 465 W/m^2. At this condition, *Azolla microphylla* had a doubling time of 3.9 d with a protein content of 30 wt%, dry basis. The highest protein recovery of 28 wt%, dry basis was obtained when *Azolla microphylla* was pre-treated with *Saccharomyces cerevisiae* for 17 h prior to extraction with sodium hydroxide (pH 12) or hydrochloric acid (pH 2).

KEYWORDS: *Azolla microphylla*; Light Intensity; Protein; *Saccharomyces cerevisiae*; Extraction;

INTRODUCTION

Azolla spp. is a small aquatic fern (1-5 cm) which has a high biomass productivity and able to grow in varied environments globally. *Azolla* spp. also has a high rate of N fixation and lives often in symbiosis with cyanobacteria *Anabena azollae* which has been extensively used for N fertilisation of paddy crops and green manure [9]. *Azolla* spp. is also known for its high protein content and can be easily cultivated for a sustainable alternative of protein source [1].

A. microphylla is among the species of *Azolla* that has the highest biomass productivity and protein content. According to the study by Arora and Singh [1], *A. microphylla* has a biomass productivity of 12 ton.ha^{-1}yr^{-1} (dry basis). The protein content of *A. microphylla* was reported to be in the range of 28- 32 wt%, dry weight as reported by Sanginga and Van Hove [11]. It was also shown in a similar study that *A. microphylla* contain a variety and high amount essential amino acids in comparison to other species with leucine (2.3 wt%, d.w.).

The protein content of *A. microphylla* may be increased by altering the light intensity during the cultivation of *A. microphylla*. Optimum light intensity is crucial for the growth kinetic and biomass development. According to a study reported by Cary and Weerts [5], increasing light intensity resulted in an increase of

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growth kinetics and biomass content of *A. pinnata* dan *A. filiculoides*. Introducing shading during the cultivation which resulted in plants receiving 30% of greenhouse sunlight reduced the biomass yield which was less than one-third those plants that were not subjected to shading. In another study by Sarkar [12], the chlorophyll, organic carbon, nitrogen and phosphorus content of *A. pinnata* were higher when the biomass was cultivated at a higher light intensity of 1200 $mol.m^{-2}s^{-1}$ than that of 700 $mol.m^{-2}s^{-1}$.

The protein content in *A. microphylla* may be extracted by using chemical solvents such acidic, basic or buffer solution or using aqueous enzymatic extraction [4]. According to the previous study by Widyarani et al [22], the extraction of protein from dehulled rubber seeds using a basic solution resulted in a protein recovery of 50-71 %. The protein yield and recovery from *A. microphylla* may also be increased by pre-treating the biomass with a biological agent during a fermentation process. According to a study by Chinma et al. [6], fermentation of wheat with yeast increased the protein yield from 10.2 to 23.3 %.

Hence, this study aimed to investigate the effect of light intensity on the growth and protein content of *A. microphylla*. The yield and protein recovery after the extraction process was also investigated by pre-treating the biomass with *S. Cerevisiae* and extraction of protein at different pH.

2. Objectives:

The objectives of this study were to investigate the effect of light intensity on the growth and protein content of *A. microphylla* and the effect of pre-treatment with *Saccharomyces cerevisiae* to increase protein recovery.

MATERIALS AND METHODS

A. microphylla plant used in this study was received in October 2015 from Cimahi, West Java, Indonesia. The received plant was cultivated and sub-cultured periodically using AB mix hydroponic medium (Cibeuniying, Bandung). *Saccharomyces cerevisiae* used in this study was obtained from a commercial yeast (Fermipan) purchased at Borma (Rancabolang, Bandung). Hydrochloric acid, sodium hydroxide, potassium phosphate buffer were obtained from School of Life Sciences and Technology, Institut Teknologi Bandung.

Cultivation of Azolla microphylla:

A. microphylla plant was cultivated in plastic containers (16.5 x 11 x 5 cm) containing 0.4 L of standard AB mix hydroponic medium [17] with a plant density of 60 g/m^2. The pH of the medium was set at 5.5 and the medium in the containers was replaced with a fresh medium every 5 d. The containers were placed under a metal frame (203 x 70 x 80 cm) covered with different type of shading materials; vinyl, polyethylene and 50% shade net that would allowed different light intensity. The plant was harvested in the morning (08.00-09.00 a.m.) and weighed before dried in an oven at 103^{O}C until a constant weight was obtained. The cultivation of *A. microphylla* was carried out for 10 d. Light intensity, relative humidity as well as bulk and medium temperature were measured three times daily (08.00 a.m., 12.00 p.m. and 16.00 p.m.) throughout the study.

Extraction of Protein:

Biomass of *A. microphylla* was shade dried for 3 d followed by oven drying at 60 ^{O}C until the moisture content of the biomass was less than 5%. The biomass was then ground using a coffee grinder and filtered with 60 mesh to produce a powdered biomass. Extraction of protein from the *A. microphylla* powder was carried out at three different pH particularly pH 2, 7 and 12. Approximately 4 g of *A. microphylla* powder was weighed and dissolved in 40 ml of either 0.1 M HCl, 0.2 M potassium phosphate buffer containing 0.5 M NaCl or 0.1 M NaOH in a 100 ml Erlenmeyer flask. The flask was mixed using a shaker at 110 rpm for 1 h at 25 ^{O}C. The mixed solution was then centrifuged twice at 1520 x *g* for 20 min before filtered with a filter paper. The supernatant was frozen using a freeze dryer.

Analytical methods:

Moisture content in the biomass of *A. microphyll* was determined by drying the sample in an oven at 103 ^{O}C overnight until a constant weight was obtained and calculated using Eq. (1).

$$\text{Moisture content (wt\%)} = (\text{Fresh weight (g)} - \text{dry weight (g)})/\ \text{fresh weight (g)} \times 100\% \quad (1)$$

The chlorophyll content of *A. microphylla* was determined using the methods suggested by Wintermans and De Mots [21]. Approximately 0.1 g fresh biomass of *A. microphylla* was ground and extracted with 10 ml of ethanol (96%) in dark conditions for 15 min. The mixture was then filtered with a filter paper (Whatman no. 1). The chlorophyll content in the filtrate was determined by measuring the absorbance at 649 and 665 nm using a spectrophotometer. The concentration of chlorophyll a, chlorophyll b and total chlorophyll was calculated using the following equation as suggested by Winterman and De Mots [21].

Chlorophyll a (mg/l) = 13.7 x A_{665} – 5.76 x A_{649}
Chlorophyll b (mg/l) = 25.8 x A_{649} – 7. 60 x A_{665}
Total chlorophyll (mg/l) = 6.10 x A_{665} – 20.0 x A_{649} (2)

where A_{649} is the absorbance at the wavelength of 649 nm and A_{665} is the absorbance at the wavelength of 665 nm . The concentration of chlorophyll in mg/l was converted into dry weight percentage using Eq. (3).

Chlorophyll content (wt%, d.w.) = chlorophyll (mg/l) / (10000 x (1-MC)) x 100% (3)

Where MC is the moisture content of fresh biomass in wt%.

The lignocellulose content of the samples was determined using a Chesson-Datta method [7]. The protein content of the sample was analyzed by Kjedahl method whereas the fat content of the sample was analyzed by using a Soxhlet extraction method (SNI-01-2891-1992). The analyses were carried out at the Analytical Laboratory, University of Padjajaran, Jatinangor.

Data analysis:

The relative growth rate (RGR) of *A. microphylla* was determined using Eq. (4) whereas the doubling time was calculated using Eq. (5).

Relative growth rate ($g.g^{-1}d^{-1}$) = ($\ln W_t - \ln W_o$)/t (4)

Where W_t is the final dry weight of the sample (g) and W_o is the initial dry weight of the sample (g).

Doubling time (d) = ln 2/Relative growth rate ($g.g^{-1}d^{-1}$) (5)

The percentage of protein recovery was calculated by comparing the amount of extracted protein with the initial amount of protein as shown in Eq. (6).

Protein recovery (%) = mass of extracted protein (g)/mass of total protein (g) (6)

The productivity of biomass was estimated based on the dry weight of *A. microphylla* obtained at the end of the cultivation period (10 d) in a container with a surface area of 0.01815 m^2.

Assuming that the biomass can be harvested every 10 d (36 times in a year) and the amount of biomass produced in a container of 0.01815 m^2 was approximately equivalent to that cultivation area of 1 ha, the biomass productivity can be estimated by Eq. (7).

Productivity of biomass ($ton.ha^{-1}yr^{-1}$) = dry weight of biomass (ton) x 10000/0.01815 (ha^{-1}) x 36 (yr^{-1}) (7)

Productivity of total protein contained in the biomass was estimated based on the total protein content in the biomass multiply with the productivity of biomass whereas the productivity of extracted protein was estimated based on the productivity of total protein multiply with the percentage of protein recovery as shown in the following equations.

Productivity of total protein ($ton.ha^{-1}yr^{-1}$) = total protein content (wt%, d.w.) x productivity of biomass ($ton.ha^{-1}yr^{-1}$) (8)

Productivity of extracted protein ($ton.ha^{-1}yr^{-1}$)=protein recovery (wt%, d.w.) x productivity of total protein ($ton.ha^{-1}yr^{-1}$) (9)

RESULTS AND DISCUSSION

Effect of light intensity on the growth and protein content of A. microphylla:

The cultivation of *A. microphylla* in this study was carried out in plastic containers containing AB mix hydroponic medium. The containers were covered with different shading materials particularly transparent vinyl, polyethylene and 50% shade net. The containers were placed in an open environment with a surrounding temperature of 27 - 32 ^{O}C as shown in Table 1. The medium temperature was slightly lower than the surrounding temperature and varied from 25 - 30 ^{O}C. These values were within the range of optimum temperature for the cultivation of *A. microphylla* which lies in the range 18 – 28 ^{O}C as reported by Tuan and Thuyet [14].

Table 1: Microclimate conditions for the cultivation of *A. microphylla* under different shading materials

Material	Surrounding temperature (^{O}C)	Medium temperature (^{O}C)	Relative humidity (%)
Vinyl	27-31	25-29	65-70
Polyethylene	28-32	25-30	62-70
Shade net 50%	28-32	25-28	61-71

A previous study by Tung and Watanabe [15] reported that exposure of *Azolla* to 30 ^{O}C (day)/29 ^{O}C (night) range decreased the heterocyst frequency of *Anabena* which affects the nitrogen-fixing ability of the association in comparison to growth at 28 ^{O}C/20 ^{O}C. Cultivation of *Azolla* at 37 ^{O}C/29 ^{O}C also caused the biomass to stop growing after two weeks and the leaves became yellowish in color [20]. The water content of *A. microphylla* cultivated under different shading materials had similar value particularly around 95 wt%. This value resembles the water content of *Azolla* spp. reported in other studies which lies in the range of 94-95 wt%. The biomass water content is influenced by the relative humidity. From table 1 it can be seen that the relative humidity for all cultivation condition resembles one another which lies in the range of 61-71 wt%. These values were slightly lower than the relative optimum relative humidity for the cultivation of *Azolla* spp. which lies in the range of 75-90 wt% [18,19]. Nevertheless, these values are still above the minimum relative humidity of 60 wt% required to avoid the biomass from becoming dry and fragile [16].

Table 2 shows the irradiance, illumination and percentage of light transmitted to the containers used for the cultivation of *A. microphylla* under different shading materials. The irradiance highly depends on the type of shading materials with vinyl plastic had the highest average irradiation (27-465 W/m^2) followed by polyethylene film (22-299 W/m^2) and shade net 50% (10-171 W/m^2). The illumination also showed similar trend which varied from 1202-44945 Lux. The irradiance and illumination fluctuated depending on the position of the sun with respect to the containers during the time of data measurement. Higher values of irradiance and illumination for containers covered under vinyl plastic can be rationalized by a higher percentage of light transmitted to the plant with an average value of 84%. The percentage of light transmitted decreased to 61% and 31% when the containers were covered by polyethylene film and shade net 50%, respectively which consequently resulted in lower values of irradiance and illumination.

Table 2: Light intensity conditions for the cultivation of *A. microphylla* under different shading materials

Material	Irradiance (W/m^2)	Illumination (Lux)	Light transmitted (%)
Vinyl	27-465	3393-44945	84
Polyethylene	22-299	2462-20661	61
Shade net 50%	10-171	1202-15813	31

Figure 1 shows the frond of *A. microphylla* after 10 d of cultivation in plastic containers containing AB mix hydroponic medium covered under transparent vinyl plastic. The width of the frond after 10 d of cultivation was approximately 1.5 cm which lies in the range of 1-5 cm reported in the literature [9]. At the beginning of the cultivation period, the weight of the biomass was approximately 0.05 g (dry weight). At the end of the cultivation period, the dry weight of the biomass was approximately 0.18 – 0.3 g which depends on the shading materials that covered the plant. The growth curve of *A. microphylla* for all types of shading materials is illustrated in Figure 2.From the figure, it can be observed that biomass growth is highly influenced by the type of shading materials.

Fig. 1: Growth curve of *A. microphylla* cultivated under different shading materials.

The relative growth rate and doubling time for all types of shading materials are presented in Table 3. The relative growth rate and doubling time of *A. microphylla* cultivated under different shading materials varied from 0.13-0.18 $g.g^{-1}d^{-1}$ and 3.9-5.4 d respectively. Different type of shading materials resulted in different amounts of irradiance received by the plants. A higher amount of irradiance resulted in a higher relative growth rate and a lower doubling time. A lower light intensity caused a lower production of photosynthate through photosynthesis and consequently inhibits growth. *A. microphylla* cultivated under vinyl plastic received a higher amount of light intensity (27 - 465 W/m^2) not only had a higher relative growth rate (0.18 $g.g^{-1}d^{-1}$) and a lower doubling time (3.9 d) but also a higher protein content.

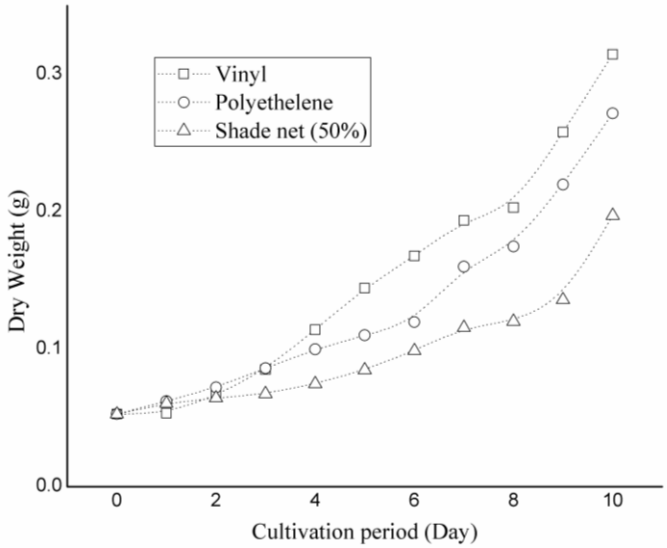

Fig. 2: Growth curve of *A. microphylla* cultivated under different shading materials.

Table 3: Relative growth rate, doubling time and protein content of *A. microphylla*

Material	Relative Growth Rate ($g.g^{-1}d^{-1}$)	Doubling time (Day)	Protein content (wt%, d.w.)
Vinyl	0.18	3.9	29.5
Polyethylene	0.16	4.2	27.2
Shade net 50%	0.13	5.4	22.2

Protein synthesis in *A. microphylla* originates from atmospheric nitrogen fixation. Nitrogenase enzyme possessed by *A. azollae* converts nitrogen in the atmosphere into ammonia which will be further assimilated into amino acid with the help of glutamine synthethase and glutamate synthetase enzyme. At a sufficient light intensity, the activity of nitrogenase during the atmospheric nitrogen fixation process is driven by adenine triphosphate that originates from the photophosphryllation cyclic process [10]. However when the light intensity is insufficient, the activity of nitrogenase enzyme depends on the amount of photosynthate in *A. microphylla* which is relatively low when the light intensity is insufficient. As such results in a lower value of protein content (22.2 wt%, d.w.) when *A. microphylla* was subjected to a lower light intensity caused by the application of shade net 50%.

Table 4 shows the chlorophyll content of *A. microphylla* cultivated under different shading materials. Chlorophyll a varied from 0.0.13 – 0.024 wt%, d.w. whereas chlorophyll b varied from 0.019-0.021 wt%, d.w. The chlorophyll a-to-b ratio was observed to increase under reduced light intensity. The total chlorophyll content increased from 0.032 to 0.044 wt%, d.w. as the light intensity reduced from 27-465 W/m^2 (vinyl) to 10-171 W/m^2 (shade net 50%). These results suggest the possibility of depression of chlorophyll content which may be due to a destruction of chlorophyll content by a higher light intensity as observed in other plants [13,8].

Table 4: Chlorophyll content of *A. microphylla* under different shading materials

Material	Total chlorophyll (wt%, d.w.)		
	Chlorophyll a	Chlorophyll b	Total
Vinyl	0.013	0.019	0.032
Polyethylene	0.015	0.019	0.034
Shade net 50%	0.024	0.021	0.044

Effect of pre-treatment with S. cerevisae on the protein content of A. microphylla:

Biomass of *A. microphylla* (obtained directly from Cimahi, West Java and not subjected to cultivation under different shading materials) that has been ground and filtered with 60 mesh was incubated with a commercial yeast (*S. cerevisiae*) for 17 h at 30 ^{O}C. Table 5 shows the composition of *A. microphylla* that has

been pre-treated with yeast in comparison to the composition without yeast pre-treatment. The protein content slightly increased from 26.5 to 27.6 wt%, d.w. when the biomass was pre-treated with *S. cerevisae*. A slight increase of protein content in the pre-treated sample may be due to the biosynthesis of amino acids by *S. cerevisiae*. Nevertheless, this value is relatively lower as compared to the protein content when *A. microphylla* was cultivated under vinyl plastic (29.5 wt%, d.w.). As such highlights the importance of suitable light intensity during the cultivation phase.

From the table, it can be observed that the lignocellulose composition for both conditions are approximately similar. As such most probably due to the absence of lignocellulosic enzymes in *S. cerevisiae* [2]. *S. cerevisae* is reported to contain an invertase enzyme that can degrade sucrose into glucose and fructose as well as maltase enzyme that can degrade maltose into two molecules of glucose [3] which may be consumed by *S. cerevisiae* as its carbon source.

Table 5: Composition of *A. microphylla* with and without pre-treatment with *S. cerevisae*

Component	Composition (wt%, d.w.)	
	Without pre-treatment	Pre-treatment with *S. cerevisae*
Ash	19.5	18.8
Crude protein	26.5	27.6
Crude fiber	16.6	15.1
Fat	2.9	1.1
Nitrogen free extractable material	34.6	37.5
Lignin	1.2	1.0
Cellulose	26.2	25.3
Hemicellulose	3.3	4.7

The effect of pre-treatment with *S. cerevisiae* on protein recovery after the extraction of protein at different pH was also investigated and the results are shown in Fig. 3. The results clearly show that pre-treatment with *S. cerevisiae* increased the protein recovery from 4-7 wt%, d.w. to 15-28 wt%, d.w. This increase is most probably due to the ability of *S. cerevisiae* to disrupt the cell wall and consequently lead to better extraction of the protein. Protein recovery is lowest at pH 7 for both samples that were not pre-treated and pre-treated with *S. cerevisiae* as illustrated in Fig. 3. Protein recovery for extraction at both pH 2 and pH 7 were approximately similar for both samples that were not pre-treated and pre-treated with *S. cerevisiae*. As such indicates that extraction of protein from the biomass of *A. microphylla* may be carried out using sodium hydroxide (pH 12) or hydrochloric acid (pH 2) and obtained similar values of protein recovery.

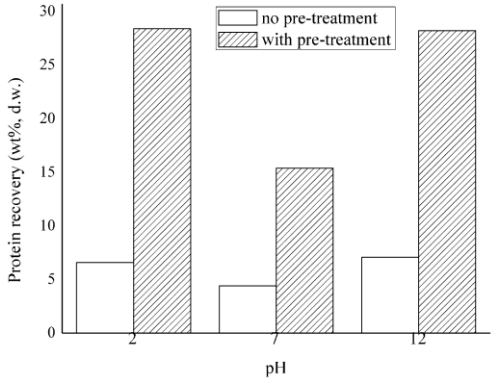

Fig. 3: Effect of pre-treatment with *S. cerevisiae* on the protein recovery after the extraction of *A. microphylla*

Estimation of productivity for biomass and extracted protein from A. microphylla:

The productivity of biomass when *A. microphylla* were to be cultivated under different shading materials varied from 3.97 to 6.33 ton.ha^{-1}yr^{-1}. From the table, it can be clearly seen that cultivation of *A. microphylla* under vinyl plastic offers higher productivity of biomass and total protein content. As discussed in the previous section, a higher amount of light intensity received by the plant (27-465 W/m^2) resulted in a higher relative growth rate and rapid atmospheric nitrogen fixation. As such results in a higher amount of biomass productivity (6.33 ton.ha^{-1}yr^{-1}) and total protein content (1.86 ton.ha^{-1}yr^{-1}) if *A. microphylla* were to be cultivated under vinyl platic as a shading material. The productivity of extracted protein were estimated based on the percentage of protein recovery (28 wt%, d.w.) when *A. microphylla* were pre-treated with *S. cerevisae* prior to extraction with sodium hydroxide (pH 12) or hydrochloric acid (pH 2). The estimated productivity of extracted protein varied from 0.25 to 0.53 ton.ha^{-1}yr^{-1}.

Table 5: Estimated productivity of biomass and extracted protein from *A. microphylla*

Material	Productivity (ton.ha^{-1}yr^{-1})		
	Biomass	Total protein	Extracted protein
Vinyl	6.33	1.86	0.53
Polyethylene	5.46	1.48	0.42
Shade net 50%	3.97	0.88	0.25

Conclusion:

The effect of light intensity to the growth and protein content of *A. microphylla* has been studied. The highest relative growth rate of 0.18 g/g.d was obtained when *A. microphylla* was cultivated using a transparent vinyl plastic with an average maximum light intensity of 465 W/m^2. At this condition, *A. microphylla* had a doubling time of 3.9 d with a protein content of 30 wt%, dry basis. The effect of pre-treatment with *Saccharomyces cerevisiae* and pH of extraction to the protein recovery was also investigated. The highest protein recovery of 28 wt%, dry basis was obtained when *Azolla microphylla* was pre-treated with *Saccharomyces cerevisiae* for 17 h prior to extraction with either sodium hydroxide (pH 12) or hydrochloric acid (pH 2).

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