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# Macrophytes to improve the cultivation of *Ankistrodesmus gracilis*

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**Abstract**
Current study investigates the effectiveness of three macrophytes as culture media on the growth of *Ankistrodesmus gracilis* in batch-culture. Microalga was grown in macrophyte culture media for 28 days and algal density was determined every day. Results showed significant difference ($p < 0.05$) in cell density, chlorophyll-*a* and lipid content with regard to *Lemna minor* when compared to other macrophyte culture media. Macrophyte culture media of *Azolla caroliniana* and *Lemna minor* were similar ($p > 0.05$) with regard to total organic carbon, total length, cell volume and protein. *Salvinia auriculata* medium had the lowest parameters. Total nitrogen in the plants were two or three times higher than total phosphorus. Although the best growth conditions for *A. gracilis* were obtained with *L. minor* culture medium, the microalga was able to grow in all macrophytes media. In fact, macrophytes are a promising tool as a culture medium due to high algal biomass, low cost and high availability in tropical countries.
**Keywords:** macrophyte medium, growth of microalgae, *Azolla caroliniana*, *Lemna minor*, *Salvinia auriculata*.

## Introduction

Aquatic plants have an important trophic role due to their high protein contents, soluble carbohydrates and reduced cell-wall fraction (Henry-Silva & Camargo, 2006). Macrophytes directly absorb nutrients and exert considerable influence on nutrients because they exude several compounds and provide colonizing sites (Polomski et al., 2007). Plants are rich in minerals and play an important role in regulating the ecological balance of the water ecosystem (Zhao et al., 2012).

The biochemical analysis of fresh water macrophytes has shown aquatic plants ability to be used as a supplementary source of food for animals and aquaculture due to their high nutrition rates arising from the richness of their biochemical constituents such as proteins, carbohydrates and lipids (Rather & Nazir, 2015).

The several uses of macrophytes are well known since aquatic plants provide many advantages, such as preventing rise in pH, reducing water temperature, curbing ammonia volatilization, suppressing weeds, habitat to many microorganisms, mosquito proliferation and others (Bhuvaneshwari & Singh, 2015). Besides harboring great number of associated microorganisms among their roots, aquatic plants are ideal component, rich in nutritional material (Sipaúba-Tavares et al., 2009).

*Azolla*, *Lemna* and *Salvinia* are genera of free-floating macrophytes. They are abundant, rich in nitrogen and protein, with a great capacity of propagation. These macrophytes are used in different ways, such as in the treatment of fish farming (Henry-Silva & Camargo, 2006), in domestic, human and pig wastes (Olguín et al., 2007), in biofuel production (Gusain & Sutta, 2017) and in bio-fertilization (Bhuvaneshwari & Singh, 2015). Some macrophytes species are being used directly in the cultivation of microalgae through the use of allelopathic substances or extract to improve growth (Zhao et al., 2012; Mendes & Vermelho, 2013). Recently, macrophytes have been directly used as culture medium to make the most of biological waste and as low cost-effective means for the production of microalgae in the laboratory (Sipaúba-Tavares et al., 2009; 2018).

*Ankistrodesmus* sp. is an important source of lipids, pigments and polysaccharides. It is also a model organism for the study of cell growth and division (George et al., 2014). The fresh water *Ankistrodesmus gracilis*, one of the live foods most frequently employed in aquaculture, may be easily cultured in the laboratory due to size, shape, thickness of cell walls, nutritional quality, easy prey conditions. It has a rapid growth rate and resists adverse conditions (Sipaúba-Tavares & Pereira, 2008).

The growth of microalgae populations depends on several abiotic factors such as light, temperature, level of nutrients (mainly nitrogen and phosphorus) that directly affect photosynthesis. Several strategies applied to improve microalgae growth, pigment and biochemical composition, include the optimization of medium composition. In fact high production costs still impair successful commercialization (George et al., 2014).

High cost of culture medium in microalgae production and extensive growth of aquatic plants restrict fishing, swimming and availability of drinking water. Consequently, the removal of these plants to improve

environmental conditions and employ them as alternative culture medium for the production of microalgae may be a low-cost tool with high nutrients content for microalga growth. Further, fish farm may also benefit from the prime matter due to the biological wastes provided. Current study examines the feasibility of employing *Azolla caroliniana*, *Lemna minor* and *Salvinia auriculata* as culture media for the growth of *Ankistrodesmus gracilis* in laboratory conditions.

**Methods**

*Microalgae strain and culture conditions*

*Ankistrodesmus gracilis* strain was obtained from culture collection 005CH, originally retrieved from the Broa Reservoir, Brazil, at 22º15' S; 47º19' W. Algae were batch-cultured at 22±2°C and exposed to light at 60 µmol $m^{-2}$ $s^{-1}$ on a 24-h light cycle. Three macrophytes, *Azolla caroliniana* (AC), *Lemna minor* (LM) and *Salvinia auriculata* (SA) were used as culture media. Culture medium with different macrophytes has already been discussed by Sipaúba-Tavares et al. (2009). The macrophytes were chosen due to their great available and abundance on the site. Table 1 shows ingredients and composition of nutrients in the culture media and wet biomass of plants. The effects of macrophyte culture media on the growth of *A. gracilis* were simultaneously investigated during a 28-day cultivation period, by experiments under the same growth conditions. Approximately 5 kg wet-weight of each plant was washed gently in tap water to remove detritus and epiphytes. They were sun-dried and then dried in an oven at 60°C, overnight. Dried plant material was homogenized in a grinder and boiled in distilled water for one hour. The hot extract was filtered and autoclaved at 120°C during 20 minutes. A 70-mL sample was collected and cooled. It was diluted with distilled water up to 1.4-L; 2.5 mL NPK were added. The experiment started with 10 mL at a density of 0.75 x $10^5$ cells $mL^{-1}$ containing $CHU_{12}$ medium. When cultures reached the last exponential growth phase ($7^{th}$ day), approximately 250 mL, a density of 2 x $10^5$ cells $mL^{-1}$, were added to the 2-L NPK medium. After the 7-day exponential growth phase, the culture density 4.4 x $10^5$ cells $mL^{-1}$ was transferred to sterilized 2-L recipients containing the three different macrophyte media. Vitamin B complex was added to the alternative culture media at the rate of 0.02 g $L^{-1}$, plus biotin (0.01 mg $L^{-1}$ (Figure 1; Table 1). Samples were analysed weekly (1, 7, 14, 21, 28 days) for growth performance and other variables during the study period.

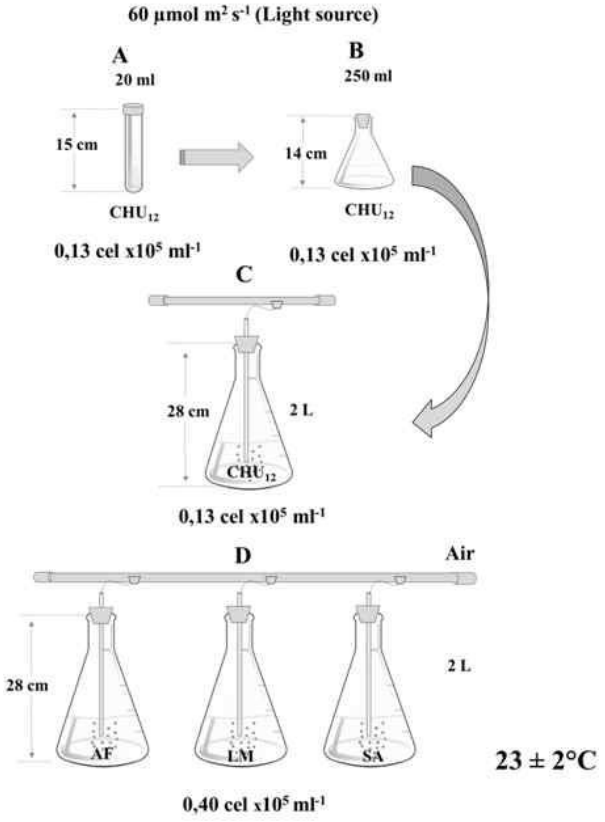


**Figure 1.** Diagram of microalga *Ankistrodesmus gracilis* in macrophyte culture media in 2L, where: A = maintenance of strain 10 mL; B = initial culture in 250 mL with $CHU_{12}$ culture medium; C = culture in 2L with NPK (20:5:20) medium; D = experiment of phototrophic system with macrophyte media of *Azolla caroliniana* (AC), *Lemna minor* (LM) *and Salvinia auriculata* (SA).

**Table 1. Nutrient composition of macrophyte media (g $L^{-1}$) and aquatic plants (g $kg^{-1}$) *Azolla caroliniana* (AC), *Lemna minor* (LM) and *Salvinia auricular* (SA).**

| Composition | Culture Media (g $L^{-1}$) | | | Plants (g $Kg^{-1}$) | | |
|---|---|---|---|---|---|---|
| | AC | LM | SA | AC | LM | SA |
| C | 1.2 | 2.4 | 0.13 | 460 | 487 | 459 |
| Ca | 0.52 | 0.55 | 2.73 | 51 | 40 | 41 |
| K | 2.27 | 2.03 | 2.0 | 33 | 26 | 18 |
| Mg | 0.17 | 0.19 | 0.08 | 8 | 6 | 6 |
| N | 0.1 | 0.3 | 0.06 | 28 | 30 | 13 |
| P | 0.05 | 0.2 | 0.06 | 8 | 9 | 7 |
| Thiamina ($B_1$) | 0.007 | 0.007 | 0.007 | - | - | - |
| Vit $B_2$ | 0.007 | 0.007 | 0.007 | - | - | - |
| Vit $B_6$ | 0.005 | 0.005 | 0.005 | - | - | - |
| Vit $B_{12}$ | 33 µg | 33 µg | 33 µg | - | - | - |
| Vit H | 0.01 mg | 0.01 mg | 0.01 mg | - | - | - |
| MO | 2.07 | 4.14 | 0.23 | 793 | 839 | 710 |
| Wet mass | - | - | - | 6,047 | 5,333 | 5,404 |
| Dry mass | - | - | - | 415 | 464 | 375 |

- = not applicable

*Microalgae growth and biochemical parameters*

Cell growth was monitored for 28-days. Triplicate 1 mL aliquots were removed daily from the microalgae culture and a minimum of 2 x 1 µL sub-samples were used for cell quantification by a Neubauer hemocytometer. Growth rate (k) was calculated by: $k = (3.322/t_2\text{-}t_1 \times \log N_2/N_1)$ (t = time; N = number of cells; subscripts denote values at different times) (Guillard, 1973). Doubling time (cell division time or generation time) was calculated from results obtained from growth rate, by: $Td = 1k^{-1}$ (Td = duplication time, $1k^{-1}$ = days per division) (Guillard, 1973). Total length of 50 specimens was determined by microscope Leica DFC 295 with image analysis system LAS core (LAS V3.8), and 40X micrometric objective. Cell volume was calculated by mean cell size with the use of the most appropriate geometric form, or rather two coupled cones formula (Hillebrand et al., 1999). Total organic carbon (TOC) was calculated by $C = 0.1204\ V^{1.051}$ (C = carbon content in pg $cell^{-1}$; V = cell volume) using regression following Rocha and Duncan (1985). Microalgae biomass was harvested, centrifuged and lyophilized for the analysis of proteins and lipids (A.O.A.C., 1990). Analyses of macro-minerals and micro-nutrients of culture media were performed weekly according to methodology by Bataglia et al. (1983). Chlorophyll-*a* was extracted with alcohol 90% and quantified at 663nm and 750nm (Nusch, 1980).

*Plants data and parameters of culture media*

Dry and wet mass of macrophytes were retrieved and dried at 60°C until constant weight and weighted. The composition of plant nutrients was analysed according to Bataglia et al (1983). Dissolved oxygen, pH and conductivity of culture media were performed weekly and measured with YSI 556 MPS multi-sensor. Total phosphorous and total nitrogen were quantified by spectrophotometry, following Golterman et al. (1978) and Koroleff (1976).

*Data analysis*

All data underwent one-way analysis of variance (ANOVA) with Statistica 8.0 package, to test the effects of the culture media (Statsoft, 2007). When differences between culture media occurred Tukey's test was applied. Differences were considered significant at $p < 0.05$. All results were expressed as means ± SD (standard deviation) and all experiments were carried out in triplicate.

**Results**

Cell concentration of *Ankistrodesmus gracilis* in macrophyte media was different ($p < 0.05$). Microalgae *A. gracilis* grew exponentially up to the 25th day in *Lemna minor* (LM) culture medium, when cell density totaled about 473 x $10^5$ cells $mL^{-1}$. After the 25th day, cell density became stable at approximately 470 x $10^5$ cells $mL^{-1}$. *Azolla caroliniana* (AC) medium showed low cell density, ranging between 5 x $10^5$ cells $mL^{-1}$ (1st day) and 292 x $10^5$ cells $mL^{-1}$ (23rd day); afterwards, cell density decreased. The growth of *A. gracilis* in *Salvinia auriculata* (SA) medium was higher than in AC medium. Highest cell density was reached on the 17th day, with 349 x $10^5$ cells $mL^{-1}$, henceforth the cell density decreased until the end of the experiment when it reached 170 x $10^5$ cells $mL^{-1}$ on the 28th day. The exponential growth of *A. gracilis* in AC and SA media occurred up to the 16th day and 14th day, respectively (Figure 2).

*Ankistrodesmus gracilis* grown in LM medium showed the highest ($p < 0.05$) lipid level and mean cell density when compared to the other culture media. However, protein level was high ($p < 0.05$) in AC and LM culture media, respectively ranging between 22% and 49% dry biomass. Total length, cell volume, total organic carbon, chlorophyll-*a* of *A. gracilis* were also high ($p < 0.05$) in AC and LM media (Table 2). Growth rate of *A. gracilis* was high in SA medium (k=0.36) due to high cell density between the 8th and 14th day, when compared to other media, ranging between 203 x $10^5$ cells $mL^{-1}$ and 303 x $10^5$ cells $mL^{-1}$; growth in AC medium ranged between 101 x $10^5$ cells $mL^{-1}$ and 167 x $10^5$ cells $mL^{-1}$ and in LM medium ranged between 124 x $10^5$ cells $mL^{-1}$ and 223 x $10^5$ cells $mL^{-1}$ (Figure 2). Consequently, doubling time of *A. gracilis* was shorter in SA medium (Table 2).

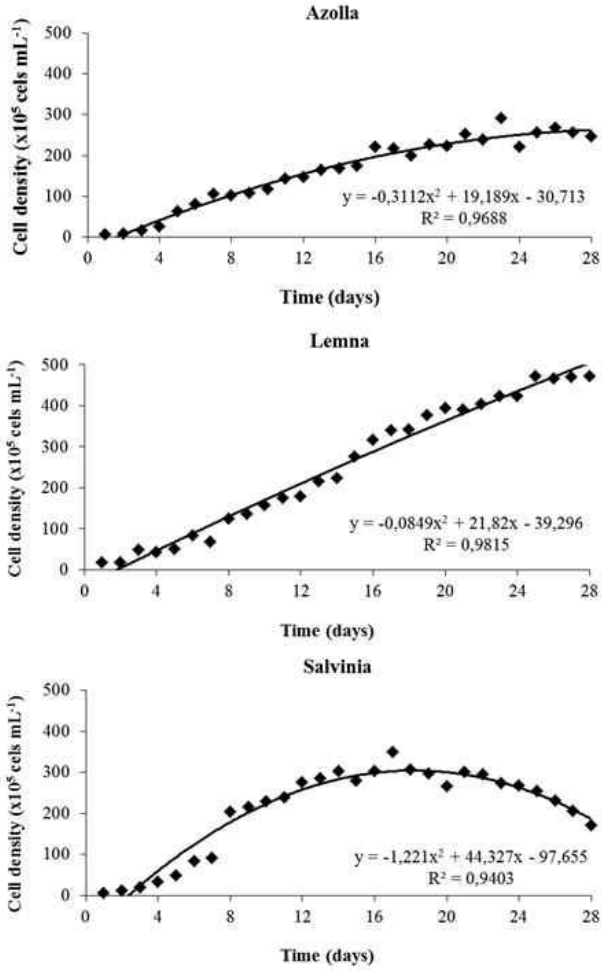


**Figure 2. Daily concentration of *Ankistrodesmus gracilis* cultured in macrophyte media of *Azolla caroliniana* (AC), *Lemna minor* (LM) *and Salvinia auriculata* (SA).**

**Table 2. Production parameters of *Ankistrodesmus gracilis* cultured in macrophytes media of *Azolla caroliniana* (AC), *Lemna minor* (LM) and *Salvinia auricular* (SA). The values are the mean of three replications and variation (±) is standard deviation.**

| Variables | Culture Media | | |
|---|---|---|---|
| | AC | LM | SA |
| Doubling time (day) | 3.8 | 5 | 2.7 |
| Growth rate (k) | 0.26 | 0.19 | 0.36 |
| Mean cell density (x$10^5$ $mL^{-1}$) | 161.8 ± 86$^{c}$ | 253.7 ± 160$^{a}$ | 208.7 ± 106$^{b}$ |
| Maximum cell density (x$10^5$ $mL^{-1}$) | 292$^{b}$ | 473$^{a}$ | 348$^{ab}$ |
| Total lenght (µm) | 19.3 ± 0.7$^{a}$ | 17.6 ± 0.6$^{a}$ | 11.8 ± 1.9$^{b}$ |
| Cell volume ($µm^3$) | 30.6 ± 5$^{a}$ | 27.3 ± 3.1$^{a}$ | 10.5 ± 3$^{b}$ |
| TOC (pg $cell^{-1}$) | 4.9 ± 1.2$^{a}$ | 4.4 ± 0.7$^{a}$ | 1.43 ± 0.4$^{b}$ |
| Chlorophyll-*a* (g $L^{-1}$) | 4.0 ± 1.4$^{ab}$ | 3.9 ± 0.5$^{a}$ | 0.4 ± 0.1$^{b}$ |
| Protein (% dry biomass) | 30 ± 11$^{a}$ | 29 ± 6$^{a}$ | 20 ± 4$^{b}$ |
| Lipid (% dry biomass) | 7 ± 2$^{b}$ | 11 ± 2$^{a}$ | 6.6 ± 3$^{b}$ |
| Dissolved oxygen (mg $L^{-1}$) | 7 ± 0.6$^{a}$ | 7 ± 0.6$^{a}$ | 7 ± 0.1$^{a}$ |
| pH | 8.7 ±1.1$^{a}$ | 8.7 ± 0.8$^{a}$ | 8.3 ± 1$^{a}$ |
| Conductivity (µS $cm^{-1}$) | 494 ± 32$^{a}$ | 468 ± 33$^{a}$ | 600 ± 169$^{a}$ |

No differences ($p > 0.05$) in water parameters of the culture media were registered. As a rule, pH was alkaline and constant air bubbling of culture medium showed dissolved oxygen above 6.4 mg $L^{-1}$. Conductivity was high, or rather, above 360 µS $cm^{-1}$. Total nitrogen was similar ($p > 0.05$) in the culture media, ranging between 0.2 mg $L^{-1}$ (LM medium) and 2.4 mg $L^{-1}$ (SA medium). Total phosphorus was highest ($p < 0.05$) in LM culture medium, between 1.1 mg $L^{-1}$ (21$^{st}$ day) and 1.2 mg $L^{-1}$ (7$^{th}$ day), and lowest ($p < 0.05$) in SA medium, between 0.3 mg $L^{-1}$ (1$^{st}$ day) and 0.4 mg $L^{-1}$ (28$^{th}$ day) (Figure 3).

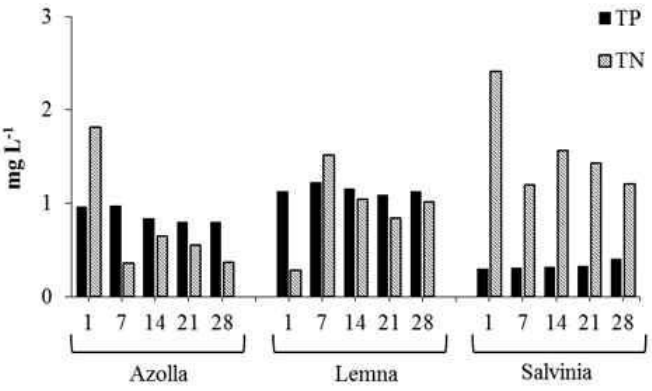


**Figure 3. Weekly variation of total nitrogen (TN) and total phosphorus (TP) in different macrophyte media of *Azolla caroliniana* (AC), *Lemna minor* (LM) and *Salvinia auricular* (SA).**

Total nitrogen in the plants were two to three times higher than total phosphorus. The composition of nutrients mainly nitrogen and potassium in the plants was high and ranged between 13 g $kg^{-1}$ (SA) and 33 g $kg^{-1}$ (AC). However, calcium was above 40 g $kg^{-1}$ and carbon had the highest concentrations, or rather, above 459 g $kg^{-1}$. Magnesium concentration was similar in LM and SA media with 6 g $kg^{-1}$. Wet mass was similar in LM and SA media and dry mass ranged between 375 g $kg^{-1}$ in SA medium and 464 g $kg^{-1}$ in LM medium (Table 2). Nutrient composition in culture media was generally above 0.05 g $L^{-1}$ (P) in AC medium, whilst K had the highest concentration in AC and LM culture media and the lowest in SA culture medium (Table 1).

**Discussion**

Low lipid levels in the *Ankistrodesmus gracilis* culture media were associated to high total nitrogen contents since lipid accumulation occurs under limited nitrogen conditions. Nitrogen deficiency causes metabolic flow of the available carbon towards fatty acid biosynthesis instead of cellular metabolism for biomass production (Singh et al., 2015).

Protein level in current study was higher than lipid level. Results were lower than those obtained by Sipaúba-Tavares et al. (2017) for *A. gracilis* using the macrophyte *Eichhornia crassipes* as culture medium with over 30% of dry biomass. Protein level is important in microalgae growth: decrease in the level of these functional proteins affects photosynthesis and amino acid synthesis, resulting in slow growth and low biomass yield (Miller et al., 2010).

When compared to protein level among macrophyte media in current study, *A. gracilis* grown in SA culture medium had the lowest protein level. Consequently, all variables related to microalgae growth were lowest. The senescent phase of growth curve was reported as from the $18^{th}$ day only in this culture medium. However, high protein level in LM medium enhanced better growth of *A. gracilis*, when compared to the other media.

The composition of different media had relevant effects on the morphology of the species *Ankistrodesmus*. The shape and size of the cells may be altered in different media (George et al., 2014). In current study, cell volume and total length in SA medium were found to be smaler than those in AC and LM media grown cell cultures.

Among various growth parameters, medium ingredients, light quality and photoperiod are the most important factors for the generation of high biomass, lipid and carbohydrate levels in microalgae (George et al., 2014). Cell density and growth rates of *A. gracilis* were influenced by the color of the culture media that in current study, changed from green to greenish yellow, while normal cell morphology may be altered. *Ankistrodesmus gracilis* grown in inorganic fertilizer (NPK) and in commercial medium ($CHU_{12}$) showed higher growth rate and cell density than the microalga AC, LM and SA cell culture media (Sipaúba-Tavares et al., 2018).

Since the lowest pigment level has been observed in cells grown in SA medium, this fact indicated lower photosynthesis rate and overall lower biomass production. Pigment composition is also affected by the color of the culture. Sipaúba-Tavares et al. (2018) observed that *A. gracilis* grown with *E. crassipes* and *E. azurea* had the same chlorophyll-a levels of cells grown in NPK culture medium. However when compared with $CHU_{12}$ commercial medium, the pigment in macrophyte culture media was higher.

Nitrogen and phosphorus levels were high in the culture media when compounds in the plants were taken into account. Total nitrogen was higher than total phosphorus only in SA culture medium. Carbon had the highest levels, followed by calcium and potassium, which are essential elements for the development of microalgae. Floating macrophytes, such as *A. caroliniana*, *L. minor* and *S. auriculata* grow up throughout the water column. Since, the roots are kept suspended in the water column all nutrients requirements for the plants must be extracted from the water. Therefore, care should be taken in choosing the place where macrophytes may yield biomass or as a culture medium for microalgae.

Recent research on macrophytes as culture medium for the growth of the species *Ankistrodesmus* has shown that each plant acts differently in biomass production. Liliopsida, the class which comprises *E. crassipes*, *E. azurea* and *Pontederia cordata* has the best growth rates and protein levels Sipaúba-Tavares et al. (2018).

Best growth conditions for *A. gracilis* in current analysis occurred with *L. minor* as culture medium. Although, growth rate, total organic carbon and protein levels in the AC culture medium were also satisfactory, not all macrophytes yielded the best results in the growth of *A. gracilis*. In current study, the microalga grew in all macrophyte media. Therefore macrophytes may be implemented as a culture medium due to their low and great availability in tropical countries. The optimization of appropriate cultivation is important so that cultivation conditions and medium composition achieve the best cell growth of microalgae. If microalga culture aims at production of biofuel, fish feed or waste treatment, the choice of macrophyte depends on the responses to protein and lipids levels. Data from current study obtained from the growth rates of *A. gracilis* cultivated in AC and LM culture media are proper to protein-based fish feed.

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