OPTIMIZATION OF A NEW CULTURE MEDIUM FOR THE LARGE-SCALE PRODUCTION OF PROTEIN-RICH ARTHROSPIRA PLATENSIS (OSCILLATORIALES, CYANOPHYCEAE)

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Abstract

Our aim was to develop a novel medium for the large-scale production of protein-rich *Arthrospira* with potential applications as a biofertilizer. The novel culture medium, termed as FM-II, was formulated using low-cost commercial chemicals and specifically designed to improve protein production. Both *Arthrospira platensis* and *Arthrospira maxima* were produced using FM-II and Arnon medium, which was used as a control. Photosynthetic status of the cells, which was checked by measuring chlorophyll fluorescence, biomass dry weight and protein content, was assessed daily. *Arthrospira platensis* had higher biomass and protein productivities than *A. maxima* when cultured in both control and FM-II media. Incorporation of varied micronutrients into FM-II formulation did not improve biomass productivity. Maximum biomass dry weight in FM-II and control medium was 2.9 and 2.5 $g \cdot L^{-1}$, respectively. Total protein content of the biomass ranged between 55% and 65%, suggesting potential for being used in the development of high-value agricultural products. As some nutrients were discarded unused, the initial content of phosphates and bicarbonates was reduced by 75% and 50%, respectively, without affecting the process productivity. Results reported herein could promote the production and utilization of *Arthrospira platensis* by significantly reducing productions costs and therefore increasing the feasibility of the process.

Keywords: *Athrospira platensis, Athrospira maxima,* marine biotechnology, microalgae, protein.

1. Introduction

The world population is expected to reach 10 billion people by 2050 (FAO 2017), doubling current agricultural demands and food production (Caporgno and Mathys 2018). Demand for protein is of special interest as protein supply is already a big concern: approximately one billion people have inadequate protein intake today (Caporgno and Mathys 2018). Animal-derived proteins are consumed in greater quantity, especially in western diets (Westhoek et al. 2014). However, livestock production is the cause of approximately 12% of greenhouse gas emissions and 30% of human-induced terrestrial biodiversity loss as well as responsible for a large share of agricultural land usage – approximately, two-thirds in the EU (Henchion et al. 2017).

Moreover, consumers are now more aware of the relationship between diet and health and the classification of red meat as Group 2A (probably carcinogenic to humans) and processed meat as Group 1 (carcinogenic to humans) by the World Health Organization (WHO) led to an increased demand for alternative protein sources. Microalgae are called to play a key role in the future food supply as these microorganisms can be used directly as food or food ingredients (Lafarga et al. 2020) or indirectly as animal feed (Dineshbabu et al. 2019) or as sources of biostimulants and biofertilizers (Renuka et al. 2018). Indeed, microalgae are one of the top trends in the food industry (Lafarga 2019), and their functional potential to promote health has been reviewed in a large number of scientific publications and books (Matos et al. 2017, Wells et al. 2017). Microalgae also show potential for being used in other high end-value applications including production of biodiesel and wastewater treatment as well as in the pharmaceutic or cosmetic industries (Spolaore et al. 2006). The most well-known and produced microalgae is Spirulina, which is a trade name given to describe mainly two species of cyanobacteria: Arthrospira platensis and Arthrospira maxima. Arthrospira species were recognized as a source of food for the future by the International Association of Applied Microbiology in 1967 (Costa et al. 2019) and are mainly known because of their high protein content, whichis around 60% on a dry weight basis (da Rosa et al. 2015). Because of their high protein content, Arthrospira species are also used to develop biofertilisers and biostimulants. Indeed, the Spanish company Biorizon Biotech SL (Almerıa, Spain) currently commercializes AlgaFert and Algafert Eco, both made with Arthrospira species. Despite the many valuable applications of microalgae, current production systems (among other challenges) are limiting the expansion of microalgae biotechnology (Garrido-Cardenas et al. 2018).

Investment in nutrients has been suggested as the second major factor influencing Spirulina production, accounting for approximately 15–25% of the total production costs (Costa et al. 2019). The main reason is the high amount of salts, such as bicarbonate, and the alkaline pH needed to grow Arthrospira species while avoiding contamination by other microorganisms. Carbon, nitrogen and phosphorous are the main nutrients required for microalgae biomass production. Carbon is (usually) supplied as arbon dioxide or as carbonate/bicarbonate. In an attempt to reduce investment in nutrients, previous studies suggested that, while supplying nitrogen and phosphorous, it is possible to substitute expensive chemicals with commercial-grade fertilizers. For example, Raoof et al. (2006) produced Arthrospira sp. using cost-effective alternative chemicals and obtained productivities that were comparable with those obtained using Zarrouks' medium, although the novel mediums' cost was five times cheaper. Similar results were reported by Madkour et al. (2012). The goal of the current paper was to formulate a low-cost culture medium for the production of A. platensis or A. maxima using commercial-

grade fertilizers. The formulated media were designed to obtain high biomass and protein productivities, as future studies will up-scale the process and use the produced biomass for designing novel foods and for the development of fertilizers rich in amino acids.

2. Materials and methods

Two different strains were studied: Arthrospira platensis (BEA 005B) and Arthrospira maxima (CCAP 1457/9). Both were kindly provided by Fundacion Cajamar (Almerıa, Spain). Selection of the most productive strain. Both Arthrospira platensis
and A. maxima were cultured indoors in 300 mL bubble-column photobioreactors with spherical bases (3-cm diameter and 45-cm height with 300-mL capacity), filled with 250 mL of culture. Experiments were performed simultaneously, and the reactors were inoculated with 10% of the culture volume from a standard inoculum. Temperature, pH, light and agitation were controlled. Three different media were used: (i) Arnon
medium supplemented with NaHCO3, which was set as the standard medium (SM) and was described in previous publications (Morales-Ama al et al. 2015); (ii) a novel medium formulated using commercial fertilizers labeled as FM-I (fertilizer medium); and (iii) the same medium formulated using fertilizers but supplemented with micronutrients, labeled as FM-IA.- The composition of these media is shown in Table 1. Temperature and pH were kept constant at 25°C and 10–11, respectively. Microalgae were grown under artificial light by fluorescence tubes of 28 W (Philips Daylight T5, Madrid, Spain), located horizontally 1 cm away from each other and 4 cm away from the culture. The irradiance inside the columns in he absence of cells was 1,850 μE m-2 s-1, measured using a spherical quantum sensor SQS-100 (Walz GmbH, Effeltrich, Germany). Light was programmed to mimic outdoor conditions:
12:12 h light:dark with a progressive increase in light intensity from 08:00 to 14:00 h and a progressive decrease from 14:00 to 20:00 h. Reactors were operated in batch mode for 6 days, after which they were operated in a semi-continuous mode. For this, every day, 30% of culture volume was harvested and replaced with fresh culture medium. Semi-continuous culture was repeated daily until the culture parameters remained constant, which meant for at least 2 weeks. Harvested b omass was used for analytical determinations. Once each experiment was concluded, the reactors were washed and re-inoculated for the next experiment, thus avoiding contaminants from previous culture conditions. Biomass dry weight and protein content were determined as described in following subsections for stationary phase cultures. Production of microalgae was performed in triplicate.

Culture medium optimization.

The composition of culture medium FM-I was improved to FM-II, shown in Table 1. In addition, the current paper also aimed at evaluating the effect of adding three different mixtures of micronutrients to the improved FM-II medium formulation, which led to three additional culture media termed as FM-IIA, FM-IIB, and FMIIC. Micronutrients A were the same oligo-elements used int he previous experiment. Micronutrients B were a mixture commercially used to promote growth of Arthrospira species and were kindly provided by Biorizon Biotech SL (Almerıa,Spain). Micronutrients C were those used in the Arnon medium. All four media were compared against SM in 300 mLphotobioreactors with pH, temperature, light, and agitate on control as described above. Each experiment was performed in triplicate. Productivity, dry weight, and protein content were determined as described in following subsections at stationary phase cultures. Next, the composition of medium FM-II was adjusted to minimize nutrient loss and the medium was reformulated reducing the concentration of bicarbonate, sulfates (magnesium), and p osphates by 50, 75, or 90%. Thus, the concentrations of MgSO4 studied were 180, 90, 45, and 18 mg L while the concentrations of KH2PO4 and NaHCO3

were 140, 70, 35, and 14 mgL-1 and 16.8, 8.4, 4.2, and 2.1 g·L -1, respectively. The effect of reducing the concentration of bicarbonate, sulfates, and phosphates of FM-II on biomass and protein productivity was assessed using 250 mL photobioreactors with pH, temperature, light, and agitation control as described above. Productivity, dry weight, and protein content
were determined as described in following subsections. All the cultures were performed in triplicate, and determinations were conducted at the end of the batch and steady-state conditions.

Analytical methods.

The composition of the culture media was determined using standard official ethods approved by the Spanish Ministry of Agriculture. These methods have been escribed and summarized in previous publications of our research group (Morales-Amaral et al. 2015). Culture media were analyzed at day 0, before inoculation, and at the end of the experiment after the biomass was harvested. All determinations were conducted in triplicate. Dry weight biomass concentration was measured gravimetrically in triplicate by filtering 50 mL of culture through 1 µm filters and drying at 70°C in an oven for 24 h. Filters were pre-dried in an oven for 24 h. Photosynthetic status of the cells was checked daily by measuring the chlorophyll fluorescence (FV/Fm) ratio with an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic). Cells
were dark-adapted for 15 min prior to measurement, and all determinations were conducted in triplicate. Protein content was determined using the Lowry method with some modifications (Lowry et al. 1951). Briefly, 25 mg of freeze-dried biomass was suspended in 150 mL of distilled water and cells were disrupted at a flow rate of
1.07 L _ h_1 using an UP400S ultrasonic processor (Hielscher, Germany) operating at 400 W and 24 kHz. Temperature was kept constant during sonication by recirculating ice-cold water. After cell wall disruption, 100 µL of sample were added to 300 µL of 1M NaOH and 700 µL of distilled water. The mixture was further mixed with 2 mL of a mixture previously prepared by adding 2 mL of 0.5% CuSO4 in 1.0% sodium-potassium tartrate plus 50 mL of 5.0% NaCO3. The mixture was vortexed and let to react in the dark. After 10 min, 400 µL of the Folin–Ciocalteus' reagent pre-diluted in distilled water (1:1; v/v) were added into the reaction and the test tubes were further let to react in the dark. After 30 min, the absorbance was read at 750 nm using a GENESYS TM 10S UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Standard curves using bovine serum albumin were prepared daily. Protein content was expressed as a percentage of the biomass dry weight.

Statistical analysis.

Results are the average of three independent experiments and were expressed as mean _ standard deviation (SD). Difference between samples was analyzed using analysis of variance (ANOVA) with JMP 13 (SAS Institute Inc., Cary, NC, USA). A Tukey pairwise comparison of the means was conducted to identify where sample differences occurred. The criterion for statistical significance was $P < 0.05$.

3. Results and discussion

Selection of the most productive strain. Zarrouk's medium successfully served as the SM for culturing Arthrospira species for many years (Zarrouk 1966). In previous studies, we observed that Arnon medium supplemented with NaHCO3 (Table 1) led to comparable Arthrospira biomass productivities, and for that reason it was used as the SM in the current study. In the first place, the current paper aimed at selecting either A. platensis or A. maxima as the most suitable strain for being grown in media formulated using agricultural fertilizers to achieve high

biomass and protein productivities. Future studies will upscale the results obtained in the current paper to large open photobioreactors and the potential of the produced biomass for being used as a biofertilizer/biostimulant in agriculture will be assessed. Results obtained for biomass concentration and protein content are shown in Figure 1. Arthrospira platensis productivity was higher than that of A. maxima in all the studied media (ANOVA; $F_{2,6} = 94.01$; $P < 0.001$). Maximum A. platensis productivity was obtained when cultured using FM-IA, calculated as 0.75 0.02 g L 1 d 1 (0.57 0.09 g L 1 d 1 for A. maxima). Moreover, A. platensis biomass productivities were higher in the culture media formulated using commercial fertilizers (FM-I and FM-IA) – when compared to the control SM (ANOVA; $F_{2,6} = 415.40$; $P < 0.001$). Indeed, biomass productivity of A. platensis in SM, FM-I, and FM-IA was 0.66 0.02, 0.74 0.04, and 0.75 0.02 g L 1 d 1 , respectively. In turn, FM-I led to lower A. maxima productivities, although no differences were observed between FM-IA and SM.

The protein content of the generated biomass is shown in Figure 1. Overall, no major differences in the protein content of the different samples were observed except for a higher protein content in Arthrospira platensis than in A. maxima when cultured in SM (ANOVA; $F_{2,5} = 19.82$; $P < 0.05$). The overall protein production of A. maxima was lower when compared to A. platensis (ANOVA; $F_{8,16} = 42.63$; $P < 0.001$). Total protein production from A. platensis in SM, FM-I, and FM-IA was calculated as 1.16 0.03, 1.32 0.04, and 1.35 0.06 g L 1 , respectively. In turn, the total protein production from A. maxima batch cultured in SM, FM-I, and FM-IA was calculated as 0.99 0.01, 0.95 0.08, and 0.97 0.06 g L 1 at day 7. The protein content of the biomass produced using FM-I or FM-IA compared well with that reported by Raoof et al. (2006), who calculated the protein production of A. platensis in the range 0.3–0.4 g L 1 when cultured in a medium formulated using cost-effective commercial chemicals. As A. maxima cultured in the fertilizer-based medium led to lower biomass and protein productivities, A. platensis was selected for further evaluation.

Optimization of culture medium. Although FM-I led to a higher biomass productivity than SM, to improve the production process, FM-I had to be modified as $NaHCO_3$ and $Ca(NO_3)_2$ formed $CaCO_3$, which precipitated and was not bioavailable for microalgal growth. For that reason, a second medium was developed, termed as FM-II. The composition of FM-II is shown in Table 1. Although $Ca(NO_3)_2$ was removed from the formulation, the total content of nitrate was not modified as it was incorporated as $NaNO_3$. Calcium was incorporated into the medium formulation as $CaCl_2$. Arthrospira platensis cultured in FM-II showed higher dry weight when compared to SM and FM-I (ANOVA; $F_{2,8} = 12.25$; $P < 0.001$). Biomass yields were comparable to those reported by Celekli and Yavuzatmaca (2009), who assessed the effect of different nitrate and salt concentrations on biomass production of Arthrospira species in Schlosser medium. In that study, the € authors reported a maximum biomass yield of 3.5 g L 1 when A. platensis was cultured in 2.5 g L 1 nitrate and 1.5 g L 1 NaCl. The higher biomass production when compared to FM-I can be attributed to the higher availability of calcium and nitrates in FM-II (because of the precipitation of $CaCO_3$ observed in FM-I) and to the higher content of nitrates in FM-II when compared to SM. Nitrate feeding was proved to affect the accumulation of phycobiliproteins, namely phycocyanin, in A. platensis previously (Manirafasha et al. 2018). Previous studies also suggested that over-compensation of nitrogen can improve the protein content of other microalgae such as Chlorella vulgaris (Xie et al. 2017). Not only the quantity but also the nitrogen source in the culture media can influence growth and composition of Arthrospira species (Colla et al. 2007). However, in the current paper, all of the designed media contained nitrates as the nitrogen source, which has been demonstrated to ensure maximum biomass yields (Rodrigues et al. 2010).

Arthrospira platensis cultured in FM-II also had a higher protein content when compared to SM and FM-I (ANOVA; $F_{2,7} = 27.54$; $P < 0.05$). This can also be partially attributed to a higher availability of nitrates. The protein content obtained in the current paper was slightly higher than that reported by Rodrigues et al. (2010), who produced biomass of A. platensis using NH_4Cl and KNO_3 as the nitrogen sources and achieved almost 50% protein content on a dry weight basis. Moreover, the effect of incorporating micronutrients into the new FM-II medium on productivity and protein content was also evaluated and results are shown in Figure 2. Overall, incorporation of micronutrients into FM-II did not affect biomass production and protein content, except for a higher protein content in FM-IIB (ANOVA; $F_{2,14} = 53.85$; $P < 0.001$). Results are comparable to those observed in the previous section of the current paper, where incorporation of micronutrients into the fertilizer-based formulation did not significantly biomass and protein productivity. A high protein content is desired, not only if the biomass is intended for agricultural applications, but also when used for the development of food and feed products. Culture media or light availability can affect protein productivity and amino acid composition (da Fontoura Prates et al. 2020). When used for food or feed, not only the protein content but also the amino acid composition of the proteins is relevant. Future studies assessing the effect of the new culture media developed herein on the amino acid composition of the biomass would be valuable for predicting the potential of using the produced biomass as an ingredient in food and feed applications.

On a following set of experiments, the utilization of nutrients was optimized. Analytical determinations suggested that some nutrients were discarded unused after harvesting the biomass. Results shown in Figure 3 as FM-II are the average of FM-II, FMIIA, FM-IIB, and FM-IIC, which were statistically equal for all the studied parameters. Overall, only approximately 20% of the sulfates and phosphates present in SM and FM-II were used for microalgal growth (Fig. 3). In addition, although nitrates were used by microalgae at a higher extent, still approximately 20% of the initial nitrates were discarded unused. FM-II showed a higher consumption of the added nutrients because of the higher biomass production when compared to SM. For this reason, as the aim of the study was to minimize culture media costs, the current experiment attempted to optimize the composition of FM-II to minimize the amount of nutrients discarded unused while maintaining high biomass and protein productivities. The effect of producing Arthrospira platensis in different concentrations of sulfates (magnesium), phosphates, and bicarbonate – representing 100, 50, 25, and 10% of their initial content in FM-II – on cell growth and protein composition, was evaluated. The initial composition of nitrates was not modified: as nitrogen is required for protein biosynthesis, limited nitrogen limits protein production (Dean et al. 2010, Ord € og et al. 2012). Nitrogen limitation can € also negatively affect growth rate and photosynthetic efficiency (Jiang et al. 2012).

Overall, biomass and protein productivity were significantly affected by the concentration of KH_2PO_4 (ANOVA; $F_{2,11} = 11.06$; $P < 0.05$) and $NaHCO_3$ (ANOVA; $F_{2,11} = 18.49$; $P < 0.001$). Reducing the content of phosphates by 50% or 75%, which would represent savings of 0.12 or 0.18 € m 3 , did not affect biomass dry weight. However, when phosphates in the medium were reduced to 14 mg L 1 , biomass dry weight at the stationary phase decreased an average of 20.7%. Reducing the content of phosphorous by 50% led to a lower protein content of the biomass. Protein content of the produced Arthrospira platensis was 51.0 0.7, 47.6 1.0, 36.1 0.1, and 36.5 2.2% when phosphates were in the media at concentrations of 100, 50, 25, and 10%, respectively. Results were in line with those reported by Markou (2012), who demonstrated that phosphorous starvation led to reduced protein (and increased carbohydrate and lipid content) in Arthrospira platensis. Although proteins and carbohydrates contain

approximately the same calorific value, Markou (2012) suggested that as proteins require approximately eight times the same number of ATPs for their synthesis than carbohydrates, and phosphorous is an essential element for ATP, phosphorous limitation affects the energy strategy of A. platensis. Results were also supported by Markou et al. (2012), who suggested that phosphorous supply could be reduced an order of magnitude with no effect on biomass production, although carbohydrate content was increased.

Moreover, Arthrospira platensis depend on sulfur for the synthesis and modification of a number of molecules. However, the mechanism of sulfur metabolism and the impact of sulfate deprivation on A. platensis are not yet fully understood. Kumaresan et al. (2017) recently reported that sulfate stress slightly (but significantly) reduced growth and down-regulated genes involved in pathways such as translation, amino acid synthesis, and protein folding, among others, and thus, they observed a reduction in the total protein content of the biomass. Furthermore, magnesium plays a key role in the photosynthetic apparatus as the center of the chlorophyll molecule as well as important functions in aggregation of ribosome into functional units, and the production of catalase (Raoof et al. 2006). In the current paper, limiting MgSO4, even by 50%, led to no microalgal growth. Although sulfates were still present at high concentrations after harvesting the biomass in control cultures (Fig. 3), limiting the availability of magnesium led to no growth because magnesium is key for the synthesis of chlorophyll and photosynthesis.

Finally, the current paper also studied the potential reduction of bicarbonate in the culture medium. Spirulina growth and production has high bicarbonate requirements, which plays a key role not only maintaining the alkaline conditions that these microalgae need, but also as a carbon source (Raoof et al. 2006). Overall, no differences in biomass dry weight were observed when NaHCO3 was reduced to 50% of the initial content (8.4 g L 1). This would represent a reduction in the culture medium price of 19.15 € m 3 . However, reducing NaHCO3 concentration to 25% or 10% (4.2 or 2.1 g L 1) led to average biomass dry weight reductions of 12.0% or 61.1%, respectively. Similar results were observed previously by Raoof et al. (2006), who reported no differences in growth, protein, and chlorophyll content of Arthrospira platensis when bicarbonate was reduced from 16 to 8 g L 1 but all three growth parameters were lower when NaHCO3 concentration was 4 g L 1 . As bicarbonate plays a role in maintaining alkaline conditions, when producing microalgae in large-scale open photobioreactors, reducing the bicarbonate content of the media could increase the risk of microbial contamination. This would need to be assessed in vitro.

4. CONCLUSIONS

Overall, Arthrospira platensis showed higher potential for being produced using agricultural fertilizers instead of expensive analytical grade chemicals (when compared to A. maxima). The goal was to produce not only high biomass productivities but also high protein productivities as the produced biomass will be further used for producing protein hydrolysates and biofertilizers rich in amino acids. The culture medium developed in the current study led to comparable biomass productivities when compared to a standard medium. However, when grown using the medium designed herein, both protein content and protein productivities were significantly higher. This would allow not only to reduce production costs by reducing significantly the cost of the culture medium, but also to potentially improve the quality of the end product, in this case, a food or agricultural product. Finally, analytical determinations suggested that both bicarbonates and phosphates could be reduced by 50% without affecting

the overall productivity of the medium. To reduce production costs will promote the production and utilization of Arthrospira platensis and open novel commercial opportunities to food processors.

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5. AUTHOR CONTRIBUTION

C. Gomez: Conceptualization (supporting), formal analysis (equal), investigation (equal), supervision (supporting), writing-review & editing (supporting). A. Guzman-Carrasco: Investigation (equal), validation (equal). T. Lafarga: Formal analysis (equal), visualization (lead), writing-original draft (lead), writing-review & editing (equal). F.G. Acien-Fernandez: Conceptualization (lead), funding acquisition (lead), project administration (lead), supervision (lead), writing-review & editing (lead).

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Figure 1

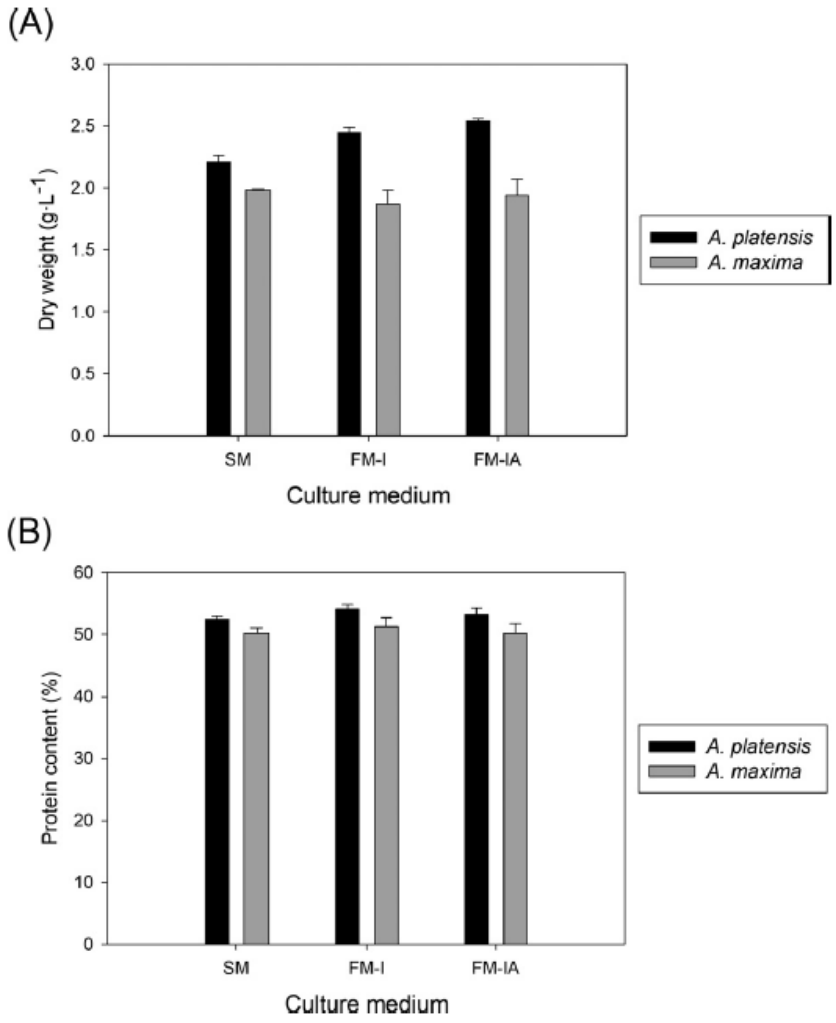

(A)
Dry weight (g·L-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
SM
FM-I
FM-IA
Culture medium
A. platensis
A. maxima
(B)
Protein content (%)
60
50
40
30
20
10
0
SM
FM-I
FM-IA
Culture medium
A. platensis
A. maxima

Figure 2

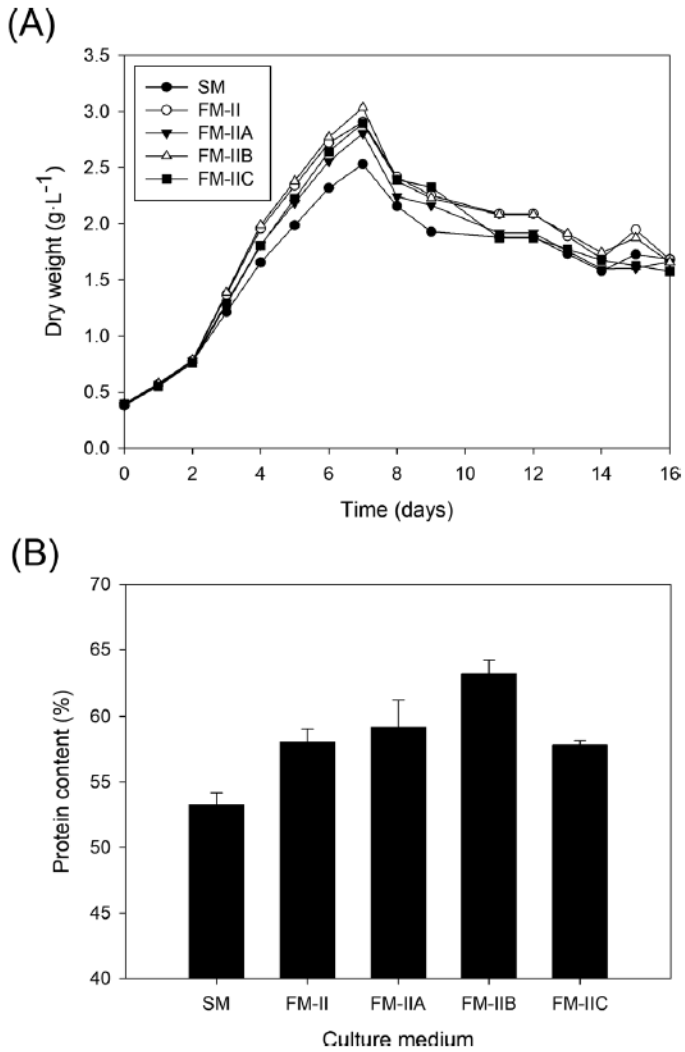
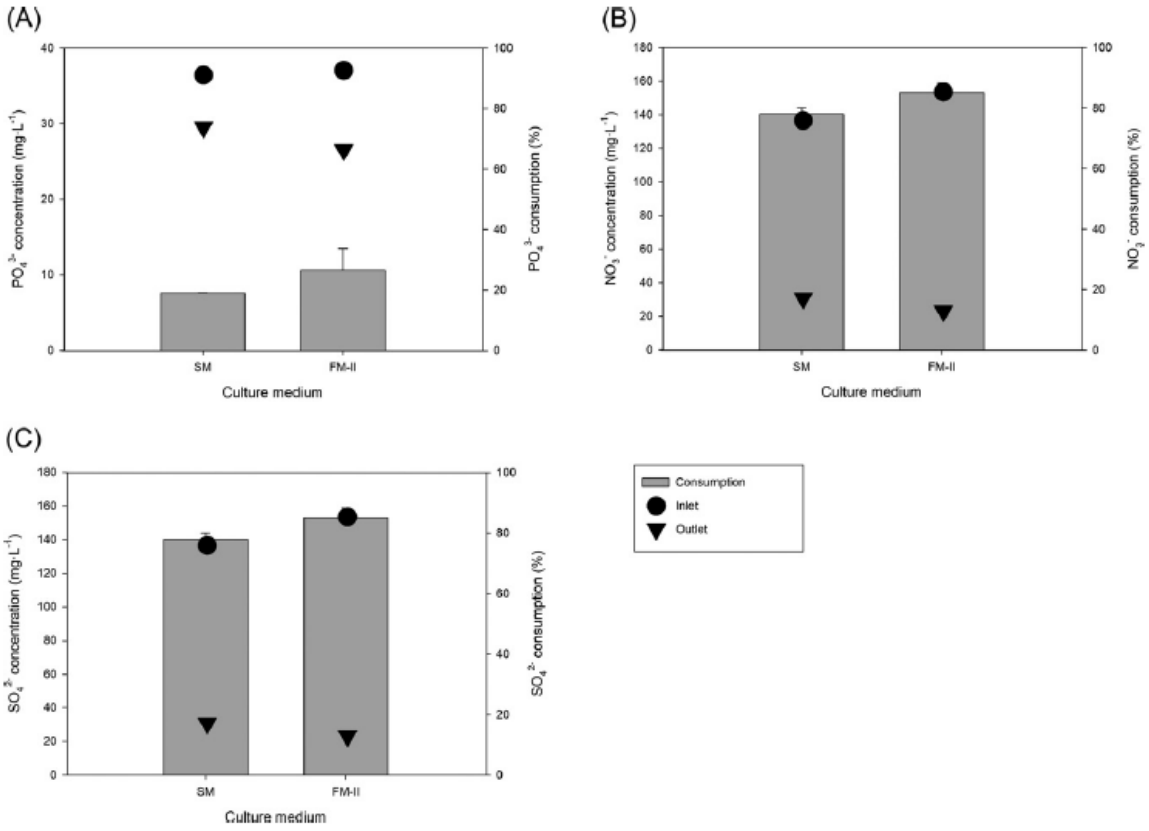

(A)
SM
FM-II
FM-IIA
FM-IIB
FM-IIC
Dry weight (g·L-1)
Time (days)
(B)
Protein content (%)
SM
FM-II
FM-IIA
FM-IIB
FM-IIC
Culture medium

Figure 3


(A)
PO_4^{3-} concentration (mg·L^{-1})
PO_4^{3-} consumption (%)
SM
FM-II
Culture medium
(B)
NO_3^- concentration (mg·L^{-1})
NO_3^- consumption (%)
SM
FM-II
Culture medium
(C)
SO_4^{2-} concentration (mg·L^{-1})
SO_4^{2-} consumption (%)
SM
FM-II
Culture medium
Consumption
Inlet
Outlet