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Consortium Growth of Filamentous Fungi and Microalgae: Evaluation of Different Cultivation Strategies to Optimize Cell Harvesting and Lipid Accumulation

Savienne M. F. E. Zorn [1,*], Cristiano E. R. Reis [1], Messias B. Silva [1], Bo Hu [2] and Heizir F. De Castro [1]

[1] Department of Chemical Engineering, Engineering School of Lorena, University of São Paulo, Lorena, São Paulo 12602-810, Brazil; cristianoreis@usp.br (C.E.R.R.); messias.silva@usp.br (M.B.S.); heizir@usp.br (H.F.D.C.)

[2] Department of Bioproducts and Biosystems Engineering, University of Minnesota, Saint Paul, MN 55108, USA; bhu@umn.edu

* Correspondence: savienne.elerbrock@usp.br; Tel.: +55-12-31595063

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Abstract: This study aims to evaluate the potential of consortium biomass formation between *Mucor circinelloides*, an oleaginous filamentous fungal species, and *Chlorella vulgaris*, in order to promote a straightforward approach to harvest microalgal cells and to evaluate the lipid production in the consortium system. A synthetic medium with glucose (2 g·L^{-1}) and mineral nutrients essential for both fungi and algae was selected. Four different inoculation strategies were assessed, considering the effect of simultaneous vs. separate development of fungal spores and algae cells, and the presence of a supporting matrix aiming at the higher recovery of algae cell rates. The results were evaluated in terms of consortium biomass composition, demonstrating that the strategy using a mature fungal mycelium with a higher algae count may provide biomass samples with up to 79% of their dry weight as algae, still promoting recovery rates greater than 97%. The findings demonstrate a synergistic effect on the lipid accumulation by the fungal strain, at around a fourfold increase when compared to the axenic control, with values in the range of 23% of dry biomass weight. Furthermore, the fatty acid profile from the samples presents a balance between saturated and unsaturated fatty acids that is likely to present an adequate balance for applications such as biodiesel production.

Keywords: fungi; algae; lichen; lipids; biofilm

1. Introduction

A bottleneck commonly reported in the viability of microalgal processes is found in the harvesting stage [1]. Although microalgae have the potential to become one of the main drivers of a new economic era due to the production of biofuels, the conditions in which microalgae are produced are still impaired by the energy intensity or the application of cost-limiting chemicals which can often cause environmental damage in the harvesting process [2]. Additional challenges are also related to the requirements of water supply to algae, in which, unlike multicellular organisms such as fungi, the cell densities obtained in conventional microalgae cultures usually present specific gravity values close to the culture medium [3]. Conventional algae harvesting and separation operations are, thus, dependent upon methods such as centrifugation, flocculation, flotation and energy-intensive filtration [2].

With the proper combination of fungi and microalgae, mimicking the natural concept of lichen, it has been demonstrated that it is possible to promote an attraction of fungal cells to microalgae,

removing almost all the microalgal species in submerged growth [4]. While the growth in a consortium system occurs for reasons of nutrient exchange and structural support, resulting in complex structures such as lichens and symbiotic biofilms, several reports in the literature indicate the possibility of the combination of algae and fungi for industrial bioprocesses [5,6]. In this sense, considering that multiple wild strains are known to produce species-specific compounds [7], the possibility of utilizing consortium growth as a synergistic approach to optimize value-added metabolites by two or more strains is, thus, interesting to the development of a robust biorefinery. The utilization of a microbial-oil-based biorefinery to produce, for instance, biodiesel or polyunsaturated fatty acids with nutraceutical value is likely to be technically possible from a process perspective utilizing the combined growth of a photoautotrophic oleaginous microalgae strain with a supporting filamentous fungal mycelium. In this line, this article addresses some technical approaches in the development of a consortium growth strategy using a fungal strain known to accumulate considerable amounts of intracellular lipids, *Mucor circinelloides* University Recife Mycologia (URM) 4182, and *Chlorella vulgaris* Banco de Microrganismos Aidar & Kutner (BMAK) D07, a microalgae species that has been widely explored in the literature regarding its oil-bearing capacity.

2. Materials and Methods

2.1. Microorganisms: Maintenance, Culture Medium, and Growth

Mucor circinelloides f. griseo-cyanus URM 4182 was selected as the fungal component for the consortium development. The URM 4182 strain was acquired from the URM Bank (Federal University of Pernambuco, Recife, Brazil) and is maintained using a Potato Dextrose Agar (PDA) medium following laboratory routine practices [8]. The microalgae *Chlorella vulgaris* BMAK D07 was donated by the Aidar & Kutner Microbial Bank (Oceonagraphic Institute, University of São Paulo, São Paulo, Brazil). The BMAK D07 strain is maintained following similar procedures as those described by Loures et al. [9].

Cell cultivation assays were performed in 250 mL Erlenmeyer flasks containing 100 mL of culture medium A [10] composed of: glucose (2 $g{\cdot}L^{-1}$)—absent in the algal axenic assays, KNO_3 (1 $g{\cdot}L^{-1}$), KH_2PO_4 (0.075 $g{\cdot}L^{-1}$), K_2HPO_4 (0.1 $g{\cdot}L^{-1}$), $MgSO_4{\cdot}2H_2O$ (0.5 $g{\cdot}L^{-1}$), $Ca(NO_3)_2{\cdot}4H_2O$ (0.0625 $g{\cdot}L^{-1}$), $FeSO_4{\cdot}7H_2O$ (0.01 $g{\cdot}L^{-1}$), yeast extract (0.5 $g{\cdot}L^{-1}$) and metal solution (1 $mL{\cdot}L^{-1}$) composed of: H_3BO_3 (2.86 $g{\cdot}L^{-1}$), $Na_2MoO_4{\cdot}2H_2O$ (0.39 $g{\cdot}L^{-1}$), $ZnSO_4{\cdot}7H_2O$ (0.22 $g{\cdot}L^{-1}$), $MnCl_2{\cdot}4H_2O$ (1.81 $g{\cdot}L^{-1}$), $CuSO_4{\cdot}5H_2O$ (0.079 $g{\cdot}L^{-1}$) and $Co(NO_3)_2{\cdot}6H_2O$ (0.049 $g{\cdot}L^{-1}$), unless specified otherwise. Algae and consortia were grown under constant light intensity equivalent to 100 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$, at 140 rpm and temperature of 26 °C. This study addresses four different development strategies of consortia, described as:

Strategy 1: 100 mL of culture medium was simultaneously inoculated with 2.55×10^8 microalgal cells and 8.5×10^5 fungal spores and incubated for 180 h.

Strategy 2: 100 mL of culture medium was simultaneously inoculated with 2.55×10^8 microalgal cells and 8.5×10^5 fungal spores and incubated for 180 h with a supporting cotton screen of 2.5 cm × 2.5 cm.

Strategy 3: 100 mL of culture medium was inoculated with 8.5×10^5 fungal spores and incubated for 72 h. After 72 h of fungal growth, 2.55×10^8 microalgae cells were added to the fungal culture, remaining in incubation until completing 180 h.

Strategy 4: 50 mL of culture medium was inoculated with 4.25×10^5 spores, and concomitantly, in another 250 mL Erlenmeyer flask, 50 mL of culture medium was inoculated with 1.275×10^8 microalgae cells. Both flasks were incubated for 72 h, followed by the transfer of the axenic cultures to a single 250 mL flask and continued incubation for 180 h.

2.2. Analytical Methods

Fungal and consortium biomass were harvested using vacuum filtration. Cell dry weight was determined via direct measurement of water loss using an infrared-coupled balance (MOC63u, Shimadzu). Glucose concentration was estimated using an adapted Dubois method [11].

The contribution of microalgal biomass in the consortium samples was determined indirectly by measuring chlorophyll-A (Chl-a) concentration present in the samples [10]. In this method, the chlorophylls present at a biomass sample with known weight and moisture content were extracted using 5 mL of aqueous methanol solution (90 vol.%) and glass beads, followed by stirring at 150 rpm at room temperature (25 °C), following by addition of 5 mL of distilled water. The liquid phase, containing the chlorophyll extract, was filtered using a 0.45 µm syringe filter. The Chl-a concentration was determined spectrophotometrically at 665 nm using a UV–VIS spectrophotometer (Varian Cary 5000), through a known correlation between algae dry weight and Chl-a absorbance at the wavelength at 665 nm. Similar measurements were made to the microalgae in suspension, i.e., to account for the microalgae cells that were not attached to the matrix in the consortium experiments or for the cell density cultures for the axenic algae assays. The fungal biomass in the consortium growth was estimated as being the difference between the consortium biomass and microalgae biomass.

Total lipids in the biomass samples were quantified by extraction performed in a digestion system with irradiation in the microwave region (CEM Discover DU-8081) using ethanol (vol. 96%) as extraction solvent, at temperature 60 °C, three cycles of 30 min [8]. The fatty acid composition was determined via methylation of lipids using a BF_3/methanol mixture following an adaptation to AOCS Ce 1-62 method. The fatty acid methyl esters (FAMEs) were identified by gas chromatography (GC) analysis using PerkinElmer®-Clarus 580 chromatograph, equipped with a flame ionization detector. A 30-m capillary column with a 0.25-mm internal diameter and 5% diphenyl 95% dimethylpolysiloxane stationary phase (non-polar) was employed during the GC analysis. Nitrogen was the carrier gas (1 mL·min^{-1}). The identification of methyl esters was carried out by comparing the retention times with MIX Supelco® Fatty Acid Methyl Acid (FAME) standard capric acid (C6: 0) to lignoceric acid (C24: 0) and quantification was performed by normalizing the calculated areas.

Attenuated Total Reflectance–Fourier Transform Infrared (ATR–FTIR) spectroscopy (Shimadzu FTIR spectrometer, model IRP PRESTIGE-21) was used to analyze the organic functions of the different consortium biomass samples. The ATR–FTIR spectra of the samples were obtained from the accumulation of a total of 32 scans at a range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}, and potassium bromide (KBr) used as a matrix.

2.3. Calculation of Biochemical Parameters

The assays were evaluated in terms of biomass and lipids, both in concentration and productivity, and in glucose consumption. The concentration of biomass, X, was determined as being the weight of the recovered biomass, which was then multiplied by the dry biomass factor, obtained via the measurement from the infrared-coupled balance, and divided by the volume used for such culture. The lipid content (% L) of each culture was determined using the Equation (1), in which m indicates the mass of either lipids or biomass (in mg), and MC is the moisture content.

$$\% \, L = \frac{m_{lipids}}{m_{biomass} \times (1 - MC)} \quad (1)$$

The productivity values of biomass (Q_X) and lipids (Q_L) were derived as being the ratio between the concentration of biomass and the cultivation time. The biomass productivity values are divided in some sections of the results as being the corresponding fungal or algal biomass. Considering that the microalgae in the flasks also can grow unattached to the consortium, this work defined the recovery efficiency of microalgae according to Equation (2), in which m indicates the dry biomass weight of the consortium in mg, w is the weight contribution, i.e., the weight of the microalgae over the total

biomass weight, [microalgae] means the concentration of algae suspended in the medium at a given time in mg·L^{-1}, and V, the medium volume in L.

$$\text{Recovery Efficiency } (\%) = \frac{m_{consortium} \times w_{microalgae}}{m_{consortium} \times w_{microalgae} + [microalgae] \times V} \times 100\% \quad (2)$$

3. Results

3.1. Axenic and Combined Cell Growth

The culture of *C. vulgaris* on medium A in the presence and absence of glucose (Figure 1a.) presented a typical growth curve and demonstrated, in fact, a similar final concentration of cells at the period of 180 h, even though a slower growth is attained in the medium supplemented with 2 g·L^{-1} within the first days of growth. Medium A, supplemented with glucose at 2 g·L^{-1}, was also able to fully support the growth of the strain URM 4182 of the filamentous fungus *M. circinelloides*, providing a quick uptake of glucose within the first 24 hours of growth, which was able to be sustained for the whole period evaluated of 180 h, as depicted in Figure 1b.

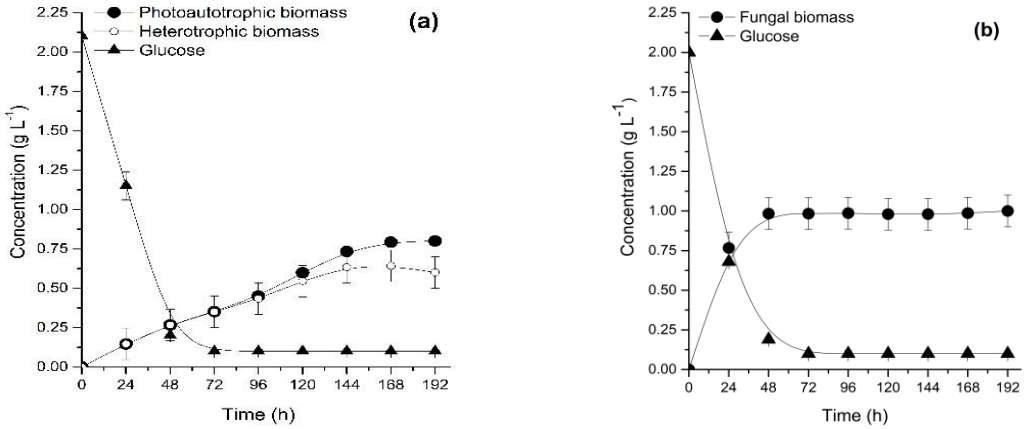
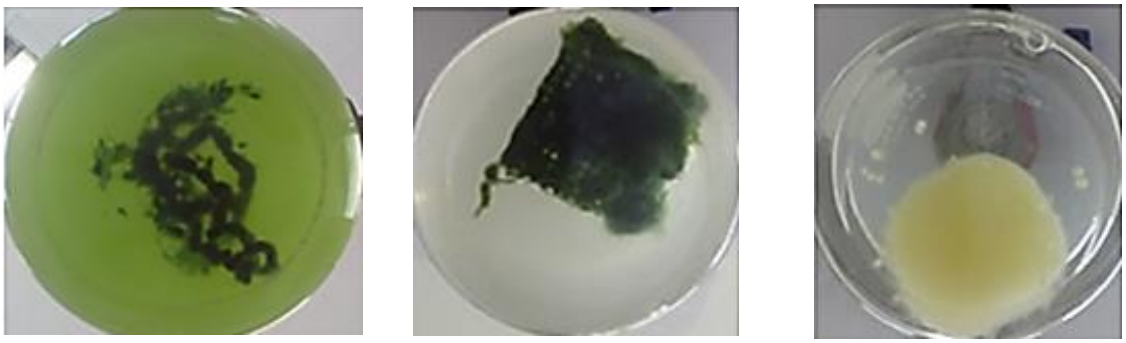


Figure 1. Growth curve of *C. vulgaris* (**a**) and *M. circinelloides* (**b**) on medium A.

The first approach considered for the recovery of *C. vulgaris* was to evaluate a simultaneous inoculation of algae cells and fungal spores, with a hypothesis that the combined growth would follow the profiles of the axenic behavior of each strain, which was named as strategy 1. The results demonstrate that the consortium system formed according this strategy was dispersed across the medium, without a clear formation of fungal pellets or other dense structures. Throughout the incubation period, a persistent and intense green color was observed in the culture medium, demonstrating that most of the microalgae cells were suspended or they did not adhere completely to the fungal biomass. Therefore, by simultaneous inoculation of cells and spores, there was poor adherence of the microalgal biomass to the fungal mycelium. The individual contributions of the consortium biomass were in average 25 ± 4% of microalgal cells and 75 ± 4% fungal mycelium. The microalgae recovery rate was within the range of 95%, demonstrating that approximately 5% of the total algal cell count was found in the liquid phase at the end of the total period analyzed. Figure 2a. illustrates in a flask the growth behavior of this strategy. Even though 95 ± 1% of algae recovery may seem like a result that would not require thorough optimization, the qualitative aspect of this given strategy demonstrates the persistence of the green color in the supernatant, which is an indication of suspended algae cells.



(**a**) (**b**) (**c**)

Figure 2. Culture flask with biomass employing strategy 1 (**a**) and employing strategy 2 (**b**). Axenic fungal mycelium in the presence of the supporting matrix used in strategy 2 (**c**).

An immobilization strategy was also taken into consideration in the evaluation of the performance of this cultivation condition, using a cotton lattice matrix as support, which was named strategy 2. The decision on testing the immobilization of the culture cells onto an external matrix was due to previously promising descriptions of algae recovery in systems assisted with fungal cells supported onto materials as cotton and polypropylene spun. The results evaluating the immobilized growth of the consortium biomass, as well as the axenic cultures of *C. vulgaris* and *M. circinelloides*, follow a similar description as described by Rajendran and Hu [10]. Both the axenic fungal culture and the consortium biomass were able to grow attached to the cotton lattice matrix, while the axenic *C. vulgaris* culture did not attach properly to the matrix. From the results it is observable a similar growth pattern as the ones without the supporting matrix in terms of biomass accumulation and glucose consumption for *M. circinelloides*.

The growth performance of strategy 2 was similar to the system without the addition of the matrix, i.e., strategy 1. The fungal mycelium was completely adhered to the cotton structure (Figure 2b) in a similar fashion to the axenic growth of Figure 2c. The composition of the consortium biomass was greater in terms of algae when compared to the non-immobilized growth (1224 mg × 1076 mg), however, the weight contribution of algae to the total biomass was still lower than 50 ± 1%, meaning that the majority of the consortium biomass was composed of fungal mycelium in terms of dry cell weight. Interestingly, on the other hand, was that the consortium biomass was greater than the axenic growth of fungal cells in the immobilized growth throughout all the cultivation process. The algal cell recovery process was also increased from the average of 95 ± 1% in the non-immobilized medium to values within the range of 99.7 ± 0.4% in the system with the cotton matrix, indicating a clear improvement in terms of algae harvesting. The microalgae in the liquid were apparently present in a smaller amount, since the color of the medium showed a much clearer aspect compared to the first approach. Due to both systems, immobilized and non-immobilized, having been inoculated simultaneously, the glucose uptake cannot be distinguished in terms of being utilized by the fungal or the algal cells. In this sense, still considering the algal cell recovery objective of this work, glucose utilization by *C. vulgaris* is unwanted, due to the fact that fungal growth, which is limited in these conditions by the sugar availability in the medium, can be partially limited by the sugar consumption by the algae cells, indicated by the lower fungal contribution to the biomass in the consortium, at approximately 560 $mg \cdot L^{-1}$, corresponding to approximately 52 ± 1% of the consortium biomass, when compared to its axenic behavior in the same medium, which achieved biomass concentrations in the range of 880 $mg \cdot L^{-1}$. In this sense, the results obtained by strategy 2 seem not to fulfill the objectives set by this work, but may represent an indication for applications in which the algal cell contribution to the total biomass is not an important to be considered, for example, those related to bioremediation of algae-contaminated systems. The adherence of the cell system to the cotton matrix could be considered as an irreversible process from a practical point of view. Therefore, the stability of

the consortia immobilized onto the matrix could be beneficial to applications in which such asset is desired, as those related to the removal of algae cells from aqueous systems, e.g., algae blooms.

3.2. Evaluation of Different Strategies for Higher Algae Recovery Efficiencies

Two additional strategies were tested in order to assay higher yields of the expected outcomes, as depicted in Figure 3. Strategy 3 was comprised of an axenic culture of *M. circinelloides*, which grew for 72 h, to which the same number of algal cells was added, leading to an overall consortium growth time of 108 h. The evolution of the results involved in this growth were based on: an initial dispersion of the algae cells onto the medium, providing a green liquid phase, followed by the fact that, approximately 24 h after the addition of *C. vulgaris* cells, the medium became clear and transparent. After 24 h, the algae cells were close to full adherence to the fungal biomass. Following the initial 24 h of combined growth, the consortium biomass presented similar total dry weight. However, the highest recovery of microalgae biomass was attained at the conditions of this consortium biomass formation, at over 99.9%, while the contribution of the algae cells to overall dry weight of the consortium biomass was within the range of 11.9 ± 1.1%. The consortium biomass, which was composed of a great majority of fungal mycelia, was greater in terms of dry weight if compared to the previous strategies, achieving values close to 2 $g \cdot L^{-1}$. Despite the fact that lower relative concentrations of algae were observed in this particular growth, it can be observed that the absolute concentration values of algae were similar to the strategies described in strategies 1 and 2, demonstrating that the early maturation of the fungal mycelia with the subsequent inoculation of algae at a lower cell count may provide a positive effect for the fungal cells, but neutral or negative growth for the algae.

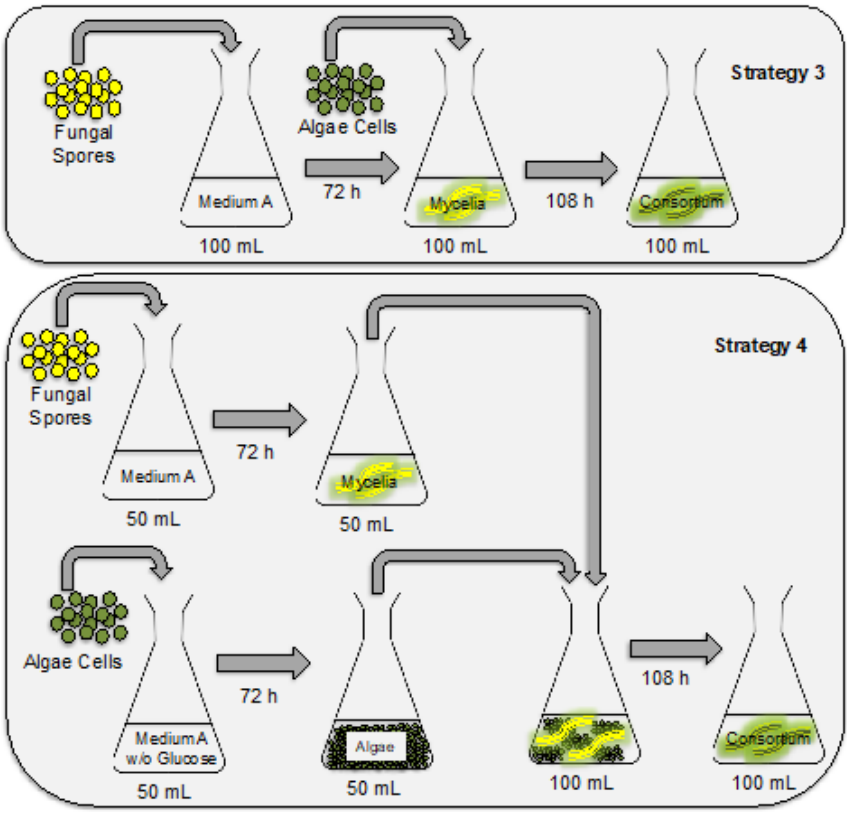


Figure 3. Schematic illustration of strategies 3 and 4.

The application of strategy 4, with a higher initial algae cell count and a mature fungal mycelium, was then tested. Microalgae cells and fungal spores were led to growth in axenic conditions for 72 h each, which were then combined, following again the overall process time of 180 h, leading to a consortium maturation time of 108 h. In this strategy, not only the fungal spores consumed the

glucose prior to the development of the consortium biomass, but the algae were able to develop in a photoautotrophic growth, achieving a concentration of approximately 350 mg·L^{-1} ± 4.1 (Figure 1a). Figure 4 summarizes the results of the strategies.

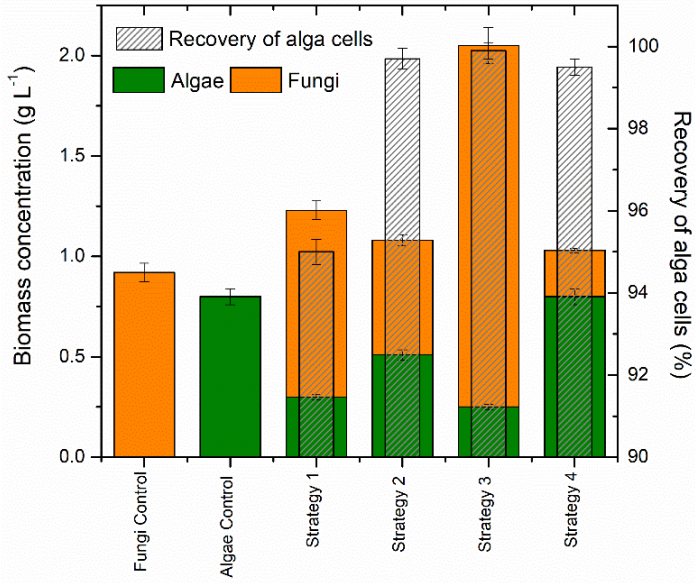


Figure 4. Summary of results according to biomass accumulation and distribution fungi (orange), algae (green) and % recovery of algae cells (grey).

3.3. *Effects on Lipid Content and Fatty Acid Distribution*

Table 1 summarizes the quantification of lipids across the strategies employed herein. The results demonstrate that, not only the total lipids are increased in all the consortium strategies when compared to the axenic cultures, but the lipid productivity of all strategies are at least 20% greater when compared to the axenic photoautotrophic growth of *C. vulgaris* under the same conditions and could be increased more than fourfold when compared to the axenic *M. circinelloides* growth on medium A.

Table 1. Summary of lipid accumulation data from different culture strategies.

Culture Condition	Total Lipids (% Dry Biomass Weight)	Total Lipids (mg·L^{-1})	Q_L (mg·L^{-1}·day^{-1})
Axenic photoautotrophic *C. vulgaris*	23.8 ± 1.0	190.4 ± 1.0	25.4 ± 1.0
Axenic *M. circinelloides*	7.1 ± 0.2	63.9 ± 0.8	8.5 ± 0.8
Strategy 1	22.7 ± 0.4	272.4 ± 0.5	36.3 ± 0.5
Strategy 2	23.5 ± 0.8	253.8 ± 1.0	33.8 ± 0.5
Strategy 3	19.0 ± 0.5	190.0 ± 0.5	22.0 ± 0.5
Strategy 4	22.2 ± 0.4	230.8 ± 0.5	30.8 ± 0.5

Even though the lipid productivity obtained by the fourth strategy was lower than counterparts 1 and 2, the consortium growth using strategy 4 was selected as the most appropriate for the objectives of cell recovery and microalgae cell contribution to the total biomass weight, as discussed in the previous sections. In this sense, a screening of process time was carried in order to evaluate the composition stability over the period of up to 384 h. The factor of a prolonged cultivation time was evaluated to assess the individualized effects on the total biomass growth, on the distribution of algae and fungi to the total biomass weight, on the microalgae cell recovery, and on the lipids, which were characterized in terms of total lipids and on fatty acid distribution. A few interesting points can be seen on the data presented in Table 2. Even though small fluctuations in the consortium dry weight were observed during the assay, the total biomass was approximately constant over the whole period.

Table 2. Summary of strategy 4 lipid accumulation at different incubation times.

Parameter	Culture Time (Days)				
	8	**10**	**12**	**14**	**16**
Consortium Biomass ($mg \cdot L^{-1}$)	1037 ± 7	806 ± 8	932 ± 7	1025 ± 7	1150 ± 6
Microalgae Recovery (%)	99.4 ± 0.2	98.8 ± 0.2	98.5 ± 0.2	98.3 ± 0.2	98.0 ± 0.2
Total Lipids (% of Dry Biomass)	31.1 ± 0.5	32.8 ± 0.5	31.6 ± 0.5	30.1 ± 0.5	30.5 ± 0.5
Fatty Acids	**Weight contribution to the total Fatty Acids (%)**				
C 12:0	1.25	1.12	1.27	1.11	1.01
C 14:0	0.91	0.87	0.90	0.76	0.71
C 15:0	6.79	6.67	6.78	7.27	7.06
C 16:0	26.58	26.79	25.56	23.81	26.25
C 17:0	1.70	1.62	1.28	2.68	2.22
C 18:0	1.31	1.29	1.63	1.79	2.03
C 16:1	0.91	0.92	0.86	0.87	0.97
C 18:1	32.98	33.06	35.0	31.34	30.07
C 18:2	11.81	11.72	12.14	11.74	11.55
C 18:3	15.76	15.93	14.57	18.64	18.13

There was no significant change in the total lipid content. The algal biomass contribution to the total consortium weight was of 79 ± 0.4%, which suggests an efficient method to obtain algae-rich biomass in coculture systems. Regarding the lipid portion of the consortium, it can be observed that the lipid content of the consortium biomass remains approximately constant throughout the evaluation period of 16 days. The fatty acid profile, as also demonstrated in Table 2, presents, at all growth times, a predominance of palmitic acid (C16: 0), ranging from 23.81 up to 26.79% in regard to the total fatty acids.

4. Discussion

4.1. Evaluation of the Culture Medium and Simultaneous Inoculation of Algae Cells and Fungal Spores

The medium selected for this study, medium A, has been described in the literature to fully support the growth of different *C. vulgaris* strains, such as UTEX 2714 [4]. The mixotrophic behavior of *C. vulgaris*, which has been widely demonstrated in multiple studies [12,13] is confirmed in this study for the strain BMAK D07. However, the growth of *C. vulgaris* in the medium supplemented with glucose was turbid and the cells were likely to be decanted, as also described by Zhang and Hu [5]. Considering that the two strains were able to grow in the same medium, the selection of the nutritional characteristics of medium A were appropriate for the evaluation of different parameters involving the interactions between the two strains.

The effect of different inoculation ratios between algae and fungi, pH, and the initial concentration of glucose, among other factors, were evaluated by Gultom et al. [4], on the interaction between *Aspergillus niger* and *C. vulgaris*, and the optimum conditions suggested by the authors were adopted for the assays involving the growth of *M. circinelloides* and *C. vulgaris* herein. Therefore, the first approach considered for the recovery of *C. vulgaris* was to evaluate a simultaneous inoculation of algae cells and fungal spores, with a hypothesis that the combined growth would follow the profiles of the axenic behavior of each strain, which was named strategy 1.

The results observed for strategy 1 indicate a potential for consortium development between the two strains, even though a higher proportion of fungal cells were observed. While there may be applications in which the composition of the consortium biomass is composed in its major proportion by fungal cells, such observation diverts from the objectives set by the current study, which are to promote recoverable biomass rich in algae cells, rather than the counterpart fungal component of the biomass.

An immobilization strategy was also taken into consideration in the evaluation of the performance of this cultivation condition, using a cotton lattice matrix as support, which was named strategy 2.

Rajendran and Hu [10] evaluated different matrices for the formation of lichen-type biofilm consisting of microalgae and filamentous fungi, resulting in a recommendation of using a polypropylene-spun lattice matrix as an efficient support for the immobilized growth of the consortium biomass. The immobilization of filamentous fungal mycelia and consortium biomass is likely to be derived from the intrinsic need for the cells to produce adhering structures to the rough cotton surface, which, despite being a complex phenomenon, is linked to the capacity of the cells to produce cellulolytic enzymes that are able to promote an initial slow degradation of the cotton matrix, from which, protein–carbohydrate structures are formed, linking the cellulose and cellular structures [14]. The results evaluating the immobilized growth of the consortium biomass, as well as the axenic cultures of *C. vulgaris* and *M. circinelloides*, follow a similar description as that described by Rajendran and Hu [10].

4.2. Other Strategies Involving the Use of Mature Fungal Mycelium

The cultivation strategy aimed for optimum results in this study should be comprised of the highest concentration of algae biomass within the consortium dry weight. Considering the results given by strategies 1 and 2, it can be observed that simultaneous inoculation may provide subpar results to the objectives set by the current study. In this sense, two further strategies were developed in order to address such issues based on the axenic separation of the fungal mycelia, with a direct consequence of depletion of glucose, which was then combined with the algae cells in order to evaluate the performance of the biofilm formation.

Strategy 3 comprised of an axenic culture of *M. circinelloides*, which grew for 72 h, to which the same number of algal cells was added, leading to an overall consortium growth time of 108 h. The evolution of the results involved in this growth were based on: an initial dispersion of the algae cells onto the medium, providing a green liquid phase, followed by the fact that 24 h approximately after the addition of *C. vulgaris* cells, the medium became clear and transparent. After 24 h, the algae cells were close to full adherence to the fungal biomass. Following the initial 24 h of combined growth, the consortium biomass presented similar total dry weight. However, the highest recovery of microalgae biomass was attained at the conditions of this consortium biomass formation, at over 99.9%, while the contribution of the algae cells to overall dry weight of the consortium biomass was within the range of 11.9%. The consortium biomass, which was composed of a great majority of fungal mycelia, was greater in terms of dry weight if compared to the previous strategies, achieving values close to 2 $g \cdot L^{-1}$. Despite the fact that lower relative concentrations of algae were observed in this particular growth, it can be observed that the absolute concentration values of algae were similar to the strategies described previously, in axenic and combined cell growth, demonstrating that the early maturation of the fungal mycelia with the subsequent inoculation of algae at a lower cell count may provide a positive effect for the fungal cells, but neutral or negative growth to the algae.

The variations in biomass contribution were evaluated according to the infrared spectrum of the different consortium samples obtained throughout the study based on the simultaneous inoculation of both fungal spores and algae cells without the influence of the cotton matrix to avoid interferences from the material used in the cell immobilization process (Strategies 1, 3 and 4). Interestingly, the ATR–FTIR spectra (Figure 5), which evaluated the functional groups in the consortium samples, presented similar profiles, demonstrating a possible similarity to the mechanism of formation, regardless of the strategy employed.

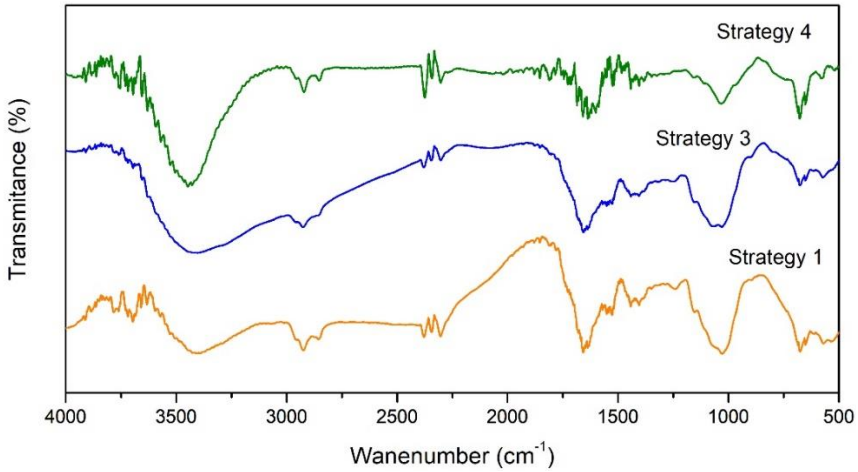


Figure 5. FTIR spectra of the consortium biomass samples from strategies 1, 3, and 4.

A thorough reading on the ATR–FTIR spectra show that, for all samples, a strong band between 3402 and 3444 cm^{-1}, the presence of -OH, likely due to the residual water activity of the samples [15]. No particular difference was either noticed within the other particular band regions of amide I and amide II, between 1480 and 1550 cm^{-1}, characterized by a particular amide C=O elongation, nor between 1444 and 1395 cm^{-1}, characteristic of vibrations of CH_2/CH_3, C=O, C-N, N-H, which constitute major protein structures (significant part of cell walls), and the C-O-C elongation vibrations between the wave numbers of 1047 to 1026 cm^{-1}, which could represent some structural difference in the polysaccharides constituting the biomass [16]. Lipids, which can be identified in similar samples by the bands related to C-H stretching vibrations [17] (=C-H stretch in the region of 3010 cm^{-1}, C-H stretching in $-CH_3$ and $-CH_2$ at 2855 and 2920 cm^{-1}) are also found in the three samples. Between 2927 and 2842 cm^{-1}, there is a characteristic fatty acid stretch with symmetrical CH_2 elongation and asymmetrical CH_2 elongation [17]. Other characteristic groups related to the presence of lipid components are the ester groups: C=O stretching at the 1740 cm^{-1} region and the C-O-C stretching (1070–1250 cm^{-1}). The C=O stretch vibrations are also characteristic to fatty acid ester bonds, which are found in all samples between 1629 and 1657 cm^{-1}.

The data presented herein are in consonance with some reports in the literature. Table 3 summarizes the strategies utilized for some recent works, demonstrating that, for the objectives of algae cell harvesting, as described herein, the utilization of a mature fungal biomass instead of simultaneous fungal spore germination and growth with the algae cells may provide better results.

Considering the multiple objectives described in the literature with the data presented herein, diverse authors describe the harvest efficiency and the composition of the consortium biomass as a determining factor for their purposes. For instance, Barnharst et al. [18] verified the application of artificial lichens using *C. vulgaris* and *Mucor indicus* in intensive aquaculture bioremediation process, observed a reduction in phosphate and total ammonia to undetectable limits, while axenic cultures were suitable for target removal of solely nitrogen or phosphorus. Rajendran et al. [22] verified the effectiveness of lichens between *C. vulgaris* and *M. circinelloides* by employing a polypropylene and cotton yarn in the ethanol co-products industry, in which the high concentration of P (818 $mg{\cdot}L^{-1}$) and N (924 $mg{\cdot}L^{-1}$) nutrients in the samples were recovered in the microalgae biomass by 55.7% and 74%, respectively, with a COD reduction in up to 65.6%. Yang et al. [20] also proposed ammonia and total solids recovery, by lichens of *Chorella* sp. and *Aspergillus* sp. in molasses wastewater, as well as achieving a microalgae recovery efficiency of over 97%.

Table 3. Comparison of microalgal cell recovery, consortium biomass and weight distribution with literature data.

Fungal strain	Microalgae Strain	Culture Medium	Immobilization Matrix	Microalgae Recovery (%)	Consortium Biomass ($mg \cdot L^{-1}$)	Microalgae: Fungal Weight Contribution to the Consortium	Reference
Mortierella isabellina, *Fusarium equiseti*, *F. lacertarum*, *Nigrospora oryzae*, *Altermaria alternate*, *F.equiseti*, *M.hiemalis*, and *M. circinelloides*	*C. vulgaris* UTEX 2714	Medium A	Polypropylene spun and tape yarn	34.85 ± 8.1–99.94 ± 0.02 (strain specific)	611.6 ± 11.9–1243.0 ± 37 (strain specific)	4.9: 95.1–48.9:51:0	Rajendran and Hu [10]
M. indicus ATCC 24905	*C. vulgaris* UTEX 2714	Medium A supplemented with ammonium concentrations ranging from 0 to 100 mg L^{-1}	Polypropylene spun and tape yarn	61.26–97.70 ($[NH_4^+]$-specific)	657.2–1125.0 ($[NH_4^+]$-specific)	19.72:80.28–80:99-19.01 ($[NH_4^+]$-specific)	Barnharst et al. [18]
A.fumigatus	*C. vulgaris*, *Chlamydomonas reinhardtii*, *Pseudokirchneriella subcapitata*, *Scenedesmus quadricauda*, *Thraustochytrid sp*, *Dunaliella tertiolecta*, *D.salina*, *Nannochloropsis oculata*, *Tetraselmis chuii*, *and Pyrocystis lunula*	Fungal growth broth with glucose or acid-pre-treated wheat straw and various dilutions of anaerobically digested swine wastewater	Self-fungal pelletization	≅ 25 -> 95 (strain and medium specific)	110–1060 (strain and medium specific)	Not specified	Wrede et al. [19]
Aspergillus sp.	*C. vulgaris*	Pre-treated molasses	Not Disclosed	>95	Up to 4125 (condition specific)	26.3:73.7–95.2:4.8 (condition specific)	Yang et al. [20]
M.elongata AG 77	*N.oceanica* CCMP 1779	Guillard F/2 medium	Not Disclosed	>60	Approximately 1000	Not specified	Du et al. [21]
M. circinelloides URM 4182	*C. vulgaris* BMAK D07	Medium A	Self-fungal pelletization	99.5 ± 0.2	1023 ± 27	79 ± 0.4: 21 ± 0.4	This work

4.3. The Effect of Different Strategies on the Lipid Accumulation and Properties

Both the microalgae and the fungal strain evaluated in this study have been reported in the literature as oil-accumulating microorganisms [23,24]. This is of interest to some biotechnological applications, including the production of biodiesel and other modifications of microbial lipids for nutraceutical applications, for example. Medium A is not an ideal growth medium intended for oil accumulation, due to an inadequate balance of carbon to nitrogen in the system, as well as a low concentration of glucose, which impairs high cell concentrations of fungi. Therefore, considering the growth of *M. circinelloides*, while the conversion of glucose to biomass was moderately high, as demonstrated in Table 1, it can be easily seen that the metabolic routes leading to oil accumulation are insufficient, attaining lipid contents in the range of 7.1 ± 0.2% in regard to the dry biomass weight in the axenic culture, whereas the same fungal strain has been reported to accumulate lipids in concentrations greater than 40% in other culture media.

The axenic photoautotrophic growth of *C. vulgaris*, however, is not dependent on sugars available in the medium [13], and, despite presenting lower biomass accumulation when compared to the fungi, the accumulation of lipids in regard to the dry biomass weight was within the range of 20% as also seen in Table 1, close to values previously reported for the same species in other photoautotrophic media [13].

The factor of a prolonged cultivation time was evaluated to assess the individualized effects on the total biomass growth, on the distribution of algae and fungi to the total biomass weight, on the microalgae cell recovery, and on the lipids, which were characterized in terms of total lipids and on fatty acid distribution. Even though small fluctuations in the consortium dry weight were observed during the assay, the total biomass was approximately constant over the whole period.

There was no significant change in the total lipid content as well. The algal biomass contribution to the total consortium weight was of 79 ± 0.4%, which suggests an efficient method to obtain algae-rich biomass in co-culture systems. The observations demonstrate a few important points, namely that the harvesting the fungal–algal system at an early time may be preferable to avoid fungal proliferation and dominance to the consortium [25,26], and the consortium system is possibly providing a cellular stress to both strains, due to the increased lipid content when compared to the axenic cultures, following a similar effect as those reported by Reis et al. [27].

Regarding the lipid portion of the consortium, it can be observed that the lipid content of the consortium biomass remains approximately constant throughout the evaluation period of 16 days. The fatty acid profile, as also demonstrated in Table 2, presents, at all growth times, a profile of fatty acids ranging from C12 up to C18, with a major concentration within the C16: 0 and C18: 1 acids (≅ 60%). It is known that the degree of saturation of fatty acids had a direct impact on the properties of the saponifiable lipids and their consequent modified products, as biodiesel. For instance, high degrees of saturation are usually linked to the greater viscosity and density of biodiesel products [28]. The abundance of unsaturated fatty acids is also known to decrease the oxidative stability of biodiesel. In this sense, it is expected that the distribution of fatty acids, which is close to some of the values also reported by Talebi et al. [28] will promote a good equilibrium between properties of flow and viscosity, which are enhanced due to the presence of unsaturated fatty acids, and oxidative stability, due to the presence of saturated fatty acids.

The distribution of fatty acids illustrated in Figure 6 demonstrates that the consortium biomass, using strategy 4 at eight days of cultivation, presents a lower contribution of saturated fatty acids when compared to the axenic controls of fungal and algal biomass using the same medium. While these results cannot distinguish between the individual contributions of fungal and algal cells and, therefore, the contributions of each species towards the characterization of the total fatty acids, the fatty acid profile obtained for the consortium presents some typical characteristics when compared to other references involving the growth of *M. circinelloides*, demonstrated by a balance between saturated and unsaturated fatty acids, as seen in Figure 6.

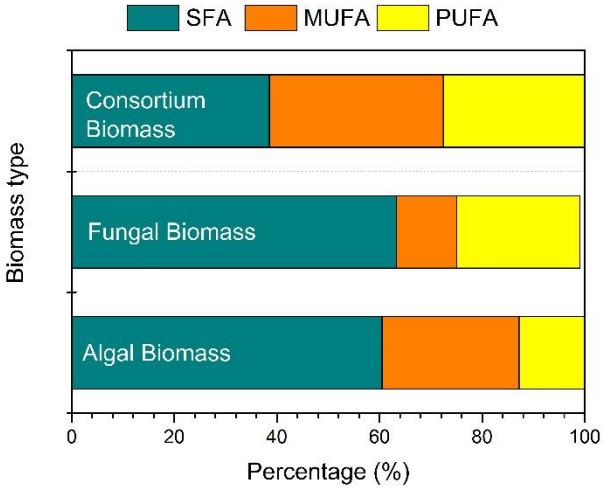


Figure 6. Distribution of fatty acids according to their category on the consortium biomass (strategy 4 at eight days of process). SFA: Saturated Fatty Acids, MUFA: Monounsaturated Fatty Acids, PUFA: Polyunsaturated Fatty Acids.

There is a wide range of factors that affect the fatty acid distribution of *M. circinelloides*. *M. circinelloides* is known to produce lipids with characteristics that are comparable to vegetable oils, thus, having been considered as a potential candidate for the establishment of microbial-biodiesel production units [6]. In fact, the stress factor related to the carbon to nitrogen ratio available in the medium regulates the content and distribution of fatty acids by *M. circinelloides*, as described by Wynn et al. [29]. As demonstrated herein, the accumulation of lipids in the consortium system has as a regulating factor, given the experimental conditions, the presence of the algae, which impairs the nitrogen availability to the fungal cells, thus promoting a higher effective carbon to nitrogen ratio, i.e., while the concentration of nitrogen is the same in both axenic and consortium systems, the consumption of nitrogen is greater in a system with two species competing for the same source of nitrogen, which induces higher accumulation of lipids by the fungi. It is important to stress that the medium selected herein has not been defined as a medium with characteristics that induce high lipid accumulation by *M. circinelloides*; in fact, this observation can be concluded by both the low lipid productivities and the rather abnormal characterization of *M. circinelloides* fatty acids when compared to the several applications in the literature [6], which often is described with relatively higher concentrations of both mono- and polyunsaturated fatty acids. The same is true for the composition of the fatty acids by *C. vulgaris*, which is often presented as a biomass rich in polyunsaturated fatty acids [30]. Therefore, the consortium biomass is likely to introduce some stress factors that induce lower accumulation of saturated fatty acids by both species and simultaneously, higher lipid productivities, possibly by decreasing the nitrogen availability to both strains involved in the culture. While there are multiple factors that affect the accumulation of lipids and the distribution of fatty acids, it can be foreseen that the application of consortium growth could induce the required characteristics for results that are likely to provide lipids with a distribution of fatty acids, as seen in Figure 6, closer to one would expect for applications in the biodiesel-production chain. In this sense, given the opportunities presented in this study, there is a potential for implementing consortium systems of algae and fungi with a possible control of the fatty acid distribution, given the stress conditions each strain is subjected to.

5. Conclusions

The article addresses an effort to recover microalgae cells using a straightforward approach based on co-cultivation with a filamentous fungus. From an initial screening of four different strategies to perform the consortium system, based on the effect of simultaneous vs. separate development of fungal spores and algae cells, and the presence of a supporting matrix, the results demonstrate that all of those attained promising initial results in the recovery of algal cells from the culture broth.

The results indicate high recovery rates, all of which are over 95%, and a synergistic effect on the lipid accumulation of both species, especially considering the fourfold increase observed on the lipid content by the axenic fungal growth when compared to the consortium system. The fatty acid distribution of the consortium system presents contributions of saturated, mono- and polyunsaturated fatty acids that could be applied for the production of biodiesel.

Author Contributions: S.M.F.E.Z., C.E.R.R. and H.F.D.C. conceived and designed research. S.M.F.E.Z. conducted experiments. M.B.S., B.H. and H.F.D.C. analyzed data. S.M.F.E.Z. and C.E.R.R. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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