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Article

Fungal-assisted microalgae for promoting growth

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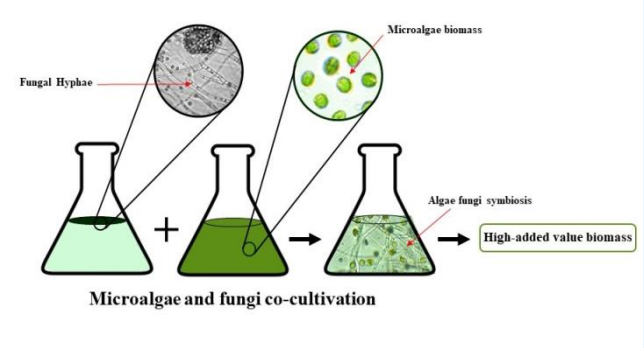
ABSTRACT: Microalgae culturing with fungal hyphae has attracted extensive consideration. This study examined the beneficial interaction between microalgae and fungi under artificial continuous illumination with fluorescent lamps of 60 μEm^{-2} s^{-1} light intensity at room temperature. The research will evaluate the algal growth dynamics at different co-cultivation times with different algal fungal loads. Highlight the species-specific dependence in terms of chlorophyll a content and biomass production. The studied microalgae encompass various characteristics, including freshwater and marine origins, motile, and non-motile properties namely *Chlorella vulgaris*, *Nannochloropsis oculata*, and *Tetraselmis chuii* and filamentous fungi *Aspergillus fumigatus*, *Rhizopus arrhizus*, and *Morterella alpina*. The growth of the symbiotic system within 72 hrs. indicated that the maximum biomass of 619.2 ± 0.97 mg/L was achieved between *C.vulgaris* and *A.fumigatus* in a ratio of 1:1. Consortia of *T.chuii* and *A.fumigatus* in a ratio of 2:1 recorded the highest chlorophyll a content of 8.16 ± 0.11 mg/L. Observing the structures of co-cultivated organisms under the light microscope validated that the microalgae cells and fungal mycelia were mutually tangled together to form the network morphology.



Abbreviations: Bold's Basal Medium (BBM); Czapek's Dox (Cz); Potato dextrose broth (PDB).

1. INTRODCTION

Organisms' interactions have been shown to regulate several life processes [1]. Different kinds of partnerships, such as mutualism, commensalism, and parasitism, may be involved in their interactions [2, 3]. These partnerships can differ based on the specific organisms as well as the environmental conditions involved in the process [4]. Numerous physical and chemical variables, such as the pH, temperature, availability of nutrients, agitation, and the ratio of algae to fungi, can affect the kind of interaction [5].

The ratio of algae to fungi is considered one of the key parameters influencing the co-cultivation process [4]. As the fungi are inoculated into the algal culture, the initial algae: fungi ratio can directly govern the growth of algae and fungi [4]. An overabundance of fungal spores might cause a preponderance of one species by competing with the algae for the limited resources in the medium [6]. To avoid negative competition and guarantee that both strains grow in balance, the inoculum ratio needs to be properly chosen [4].

The co-cultivation technology of algal fungal strains is primarily influenced by the strain selection of microorganisms [4], as it directly determines the co-cultivation efficiency and affects the microalgal biomass production [6]. Bhattacharya et al [7].

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Demonstrated the importance of the algal strain selection by using *Aspergillus fumigatus* with various microalgal biomass. Algae/fungi interaction can be attributed to microalgal-specific characteristics, such as size, shape, cell wall biochemical composition, and hydrophobicity [8, 9]. Additionally, the unique algal strain properties contribute to the variations in the performance of co-cultivation and subsequent efficient biomass production [4].

Co-cultivation time is also considered a factor that can influence the algal-fungal co-culture system. The optimal cultivation time depends on the strains used [10]. At the same time achieving maximum growth which is essential during microalgae biomass production is governed by culture time [11]. Arora et al. [11] stated that the maximum growth is determined by the culture time during biomass harvesting. So co-cultivation period has an important impact on the algal-fungal co-culture system [10].

According to Phang et al. [12], algae are adaptable creatures with a vast array of applications. These are photosynthetic organisms that produce a great amount of atmospheric oxygen through photosynthesis by which solar energy is transformed into chemical energy [13]. Algae can create biomass which can be utilized to produce biofuel and fix carbon dioxide [2]. Pharmaceuticals, nutraceuticals, cosmetics, and aquaculture products are just a few of the biotechnological uses for algae [13, 14]. In the context of integrated biorefineries, algal fungal co-cultivation is a potential technology since it creates opportunities for the generation of valuable bioproducts from the harvested biomass [15].

The synergism effect on biomass production was demonstrated by algal-fungal co-cultivation. It has a synergistic effect on biomass production and holds promise for producing bio-based chemicals sustainably [16]. According to Lutzu and Dunford [2] the presence of microorganisms with microalgae has been shown to improve the chemical composition of algal cells and greatly increase their growth rate, productivity, and biomass production [17].

To optimize the sustainability benefits of algae/fungi co-cultivation, research and development efforts must be sustained to enhance the processes' scalability, efficiency, and environmental performance [15].

The main emphasis of this study is to highlight the potential benefits of co-cultivation on microalgal growth. Fungal-assisted microalgal biomass production will be investigated to address: (1) Algal-fungal species dependence (2) Determine the ideal ratios of co-partners (3) Co-cultivation time optimization (4) Achieve the highest possible microalgal biomass production. Microalgae *Chlorella vulgaris*, *Nannochloropsis oculata*, *Tetraselmis chuii* along with the filamentous fungi *Aspergillus fumigatus*, *Rhizopus arrhizus*, *Morterella alpina* were selected.

2. Materials and methods

2.1. Microalgae and culture conditions

Freshwater microalga *C.vulgaris* was initially obtained from Phycology Laboratory's Culture Collection at Alexandria University, Faculty of Science. And cultivated in Bold's Basal Medium (BBM) [18], with the following formula. K_2HPO_4 (0.075 g/L), KH_2PO_4 (0.014 g/L), $MgSO_4.7H_2O$ (0.075 g/L), $NaNO_3$(0.09 g/L), $CaCl_2.2H_2O$ (0.025 g/L), NaCl (0.025 g/L), EDTA-Na_4 (0.05 g/L), $FeSO_4.7H_2O$ (0.00498 g/L), H_3BO_3 (0.01142 g/L), $MnCl_2.4H_2O$ (0.232 mg/L), $ZnSO_4.7H_2O$ (1.41 mg/L), $CuSO_4.5H_2O$ (0.252 mg/L), $NaMoO_4.5H_2O$ (0.192 mg/L) and $CoCl_2.6H_2O$ (0.080 mg/L).

The marine microalgae *T.chuii* and *N.oculata* were obtained from Algae Biofuels and Biorefinery Laboratory's Culture Collection at Alexandria University, Faculty of Science. According to Pokorny et al. [19], the algae were axenically grown in enriched Mediterranean seawater (El Shatby, Alexandria, Egypt, GPS position: 31.2137° N, 29.9179 E) F/2 medium [20], comprising KNO_3 (0.0750 g/L), $NaH_2PO_4.2H_2O$ (0.00565 g/L), EDTA-Na_2 (0.00436 g/L), $FeCl_3{\cdot}6H_2O$ (0.00315 g/L), $CuSO_4{\cdot}5H_2O$ (0.00001 g/L), $ZnSO_4{\cdot}7H_2O$ (0.000022 g/L), $CoCl_2{\cdot}6H_2O$ (0.00001 g/L), $MnCl_2.4H_2O$ (0.00018 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.000006 g/L), cyanocobalamin (B12) (0.000002 g/L), thiamine (B1) (0.0001 g/L) and biotin (0.000001 g/L).

All chemicals were purchased from El-Gomhouria Pharmaceutical Company, Alexandria, Egypt.

The pH was adjusted to 8 before autoclaving for 15 min at 121°C. A 400 ml media was inoculated with microalgal cells ($4*10^6$ cells ml^{-1}) using three replicates in a 1 L Erlenmeyer flask. Through standardized batch cultures, algae were incubated in laboratory conditions at room temperature under artificial continuous light with fluorescent lamps set to 60 μEm^{-2} s^{-1} intensity. Handshaking was used to provide aeration.

2.2. Culturing of filamentous fungi

The three strains of fungi *A.fumigatus*, *R.arrhizus*, and *M.alpina* were provided by Assiut University Moubasher Mycological Centre in Assiut, Egypt. *A.fumigatus* was cultured on Czapek's Dox (Cz) media and its composition was defined by Thom and Raper [21], while *M.alpina* and *R.arrhizus* were cultured on Potato dextrose broth (PDB) media, which described by [22]. Before sterilization, the pH was adjusted to 7.0 for both media.

2.3. Co-cultivation experimental setup

Fungi species were sub-cultured in potato dextrose and Czapek's Dox broth medium from four to seven days before co-cultivation [23]. Then the fungal mycelia were filtered then cleaned with sterile algal media and deionized water before being mixed with microalgal cultures [24]. Wet fungal mycelia were combined with algal cultures [25]. By adjusting the immobilization time (24–72 hrs.) and the fungal mycelium load in ratios of 1:1 and 2:1 alga/fungus (v/v). The algal-fungal co-culture was incubated for three days at room temperature (22–23°C) under continuous light (60 μEm^{-2} s^{-1}) without being shaken [26]. According to Talukder et al. [25], every trial was biologically averaged from three replicate studies.

2.4. Growth Measurements

Growth was determined according to cell count, biomass concentration, and chlorophyll a content.

2.4.1. Cell Counting

Using an optical microscope (OPTIKA light microscope-Italy Mi5200 Software), direct microalgal count was carried out using a haemocytometer (Neubauer brand) [27]. The equation stated by Al-Rubeai et al. [28] was used to obtain cell density :

Cell density = Average number of cells * 10^6/ml

2.4.2. Biomass determination

Biomass was measured as biomass concentration and productivity. To evaluate the growth of the cells as biomass concentration, the absorbance of each culture in three replicates was measured at 625 nm in a UV-2005 Selecta spectrophotometer, and a corresponding blank medium without algae was used as the control. The average value was recorded.

According to Sánchez Mirón et al. [29], the biomass concentration was calculated:

Biomass concentration (mg/L) = 0.38 * Optical density at 625 nm

2.4.3. Chlorophyll measurement

Chlorophyll a measurement was performed as previously described by Jeffrey [30]. The samples were measured spectrophotometrically using a UV-visible spectrophotometer (UV-2005 Selecta) at 663.6 nm and 646.6 nm counter to the solvent (acetone) as blank. Porra [31] proposed the following equation to estimate the chlorophyll a concentration in mg/L:

Chlorophyll a concentration (mg/L) = 12.25 ($A_{663.6}$) - 2.55 ($A_{646.6}$)

2.5. Microscopy

Using an optical microscope (OPTIKA–Mi5200 Software–Italy) with an attached camera (Optix Cam), the morphology of the microalgal cells and algae/fungi co-cultivation system were observed.

2.6. Statistical analysis

All data were expressed as mean values of three replicas ± standard errors. To estimate the statistically significant differences, a three-way analysis of variance with Post Hoc Test (Tukey) ($P \leq 0.05$), using SPSS version 25, IBM Corporation was employed for all analyses.

3. Results and discussion

3.1. Controlled conditions

(Figure 1) demonstrates the growth profile of microalgae measured as cells number. The growth showed a typical pattern with phases comprising adaptation, logarithmic, and stationary. Although an equal initial inoculum was used for these species. There were noticeable differences between them in terms of cells number. *T.chuii* showed the largest number throughout the cultivation period, starting from 10. 33 ± 1.53 *10^6 cells/ml on the 2nd day and reaching 39.33 ± 0.58*10^6 cells/ml on the 24th day. While *N.oculata* and *C.vulgaris* were the lowest in the number of cells, the readings recorded 8.33 ± 0.58*10^6 and 5.33 ± 1.53*10^6 cells/ml at the beginning until it reached a maximum number of 31 ± 1*10^6 and 25*10^6 cells/ml at the end of the experiment respectively. During the lag phase and initial adaptation to a new environment, there was no marked significant growth, algal cells may not exhibit cell division as they are adjusting to the new conditions and preparing for active growth [32]. Once fully adapted, the log phase begins. In this phase, cells multiply rapidly, they uptake nutrients to support their growth and obtain the energy leading to exponential growth and the number of cells increases over time [33]. Eventually, in the stationary phase, resources become limited, and growth levels off, where algae continue to divide, but the rate of cell division equals the rate of cell death and stagnant growth is marked [34]. Consequently, harvesting algal cultures during the early stationary phase with high cell density and biomass is recommended.

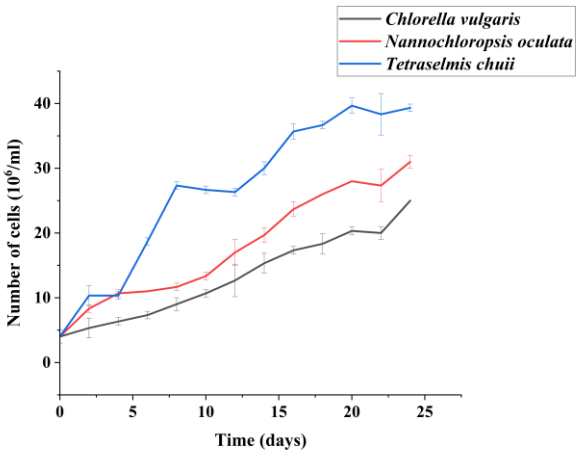

Figure 1. Cells number of *Chlorella vulgaris*, *Nannochloropsis oculata*, and *Tetraselmis chuii*.

(Data are expressed as the means of three replicates and the standard deviation is indicated by error bars).

(Figure 2) shows chlorophyll a content in the examined microalgae, it can be noticed that both *C.vulgaris* and *N.oculata* were close in their chlorophyll a content, while *T.chuii* have the highest level. The chlorophyll a content of *T.chuii* achieved 4.650 ± 0.050 mg/L. It is two times higher than those of *C.vulgaris* (1.133 ± 0.029 mg/L) and five times higher than the concentration obtained with *N.oculata* (0.753 ± 0.047mg/l). Chlorophyll molecules play a critical role in photosynthesis and are essential indicators of reflecting growth activity [35]. Increasing chlorophyll a content to the maximum reflected that the microalgal activity is strong which was mainly involved in promoting the photosynthesis process. The chlorophyll a content and biomass concentration of different microalgal species can vary even under the same growth conditions, as algae species exhibit unique physiological and biochemical traits [36]. Some species are inherently better at capturing nutrients, utilizing light, or storing energy [37]. These traits influence their growth rates, chlorophyll a content, and biomass production [38]. Also, algae differ in their ability to take up nutrients from the environment. Efficient nutrient uptake leads to higher biomass accumulation [39]. Furthermore, algae have different light requirements, light availability affects chlorophyll a production and, consequently, biomass production [40]. The type of culture medium used, such as the carbon and nitrogen sources, can influence the growth, lipid, and pigment production in co-cultures of microalgae and fungi. Optimizing the culture medium composition is important for enhancing the desired metabolite production [41].

Results recorded in Table 1 indicated that there was a gradual increase in biomass concentration as a function of time, starting on the first day (0 time) from 34.24 ± 0.64, 68.93 ± 0.23, and 54.70 ± 0.45 mg/L to algae *C.vulgaris*, *N.oculata* and *T.chuii* respectively. *C.vulgaris* significantly achieved the highest

biomass concentration (250.43 ± 13.5 mg/L) compared to *T.chuii* and *N.oculata* (193.03 ± 1.95 and 191.14 ± 3.77 mg/L) respectively after 24 days of cultivation. In the first 48 hrs., the growth was almost slow which was the growth delay period. At this period, the biomass displays an equilibrium stage. Microalgae are adapted to conditions by manufacturing the essential metabolites and maintaining their life activities [42]. After that, growth was very fast and increased exponentially. During this stage, the rapid growth of biomass ensures the stability of the system.

Table 1: Biomass concentration (mg/L) in fed-batch culture of *Chlorella vulgaris*, *Nannochloropsis oculata*, and *Tetraselmis chuii*

	Biomass concentration (mg/L)		
Time /days	*C.vulgaris*	*N.oculata*	*T.chuii*
0	34.24 ± 0.64	68.93 ± 0.23	54.70 ± 0.45
2	38.37 ± 1.58	78.66 ± 3.21	97.28 ± 2.53
4	50.54 ± 2.21	87.97 ± 2.63	114.75 ± 5.08
6	62.70 ± 2.52	97.28 ± 2.53	120.08 ± 5.0
8	87.78 ± 2.55	101.84 ± 7.41	133.0 ± 1.73
10	104.88 ± 1.10	114.37 ± 1.52	139.07 ± 3.04
12	121.98 ± 9.88	126.54 ± 3.0	144.78 ± 5.01
14	137.56 ± 8.91	153.52 ± 5.62	159.60 ± 5.05
16	165.30 ± 5.03	159.60 ± 4.41	161.12 ± 3.46
18	179.55 ± 5.69	175.0 ± 5.0	171.0 ± 2.65
20	193.81 ± 1.36	164.15 ± 1.88	175.17 ± 1.06
22	218.49 ± 1.87	179.73 ± 1.30	177.47 ± 1.50
24	250.43 ± 13.50	191.14 ± 3.77	193.03 ± 1.95

Chlorella vulgaris was grown in BBM [18] and *Nannochloropsis oculata* and *Tetraselmis chuii* were grown in F/2media [20]. Biomass concentrations were measured at a light wavelength of 625nm.

The data shown are the average biomass concentrations from three biological replicates. Expressed using mean ± standard deviation.

3.2. Growth characteristics of symbiotic systems

Compared to pure algal cultures, (**Figure 3**) showed a significant increase in the chlorophyll a content of microalgae along with all consortia conditions. Concerning *C.vulgaris* when cultured in symbiosis with fungal co-partners (**Figure 3A**), chlorophyll a content (mg/L) was calculated. At the beginning and at zero-time symbiosis, it recorded 0.92 ± 0.03 mg/L. After 72 hours of co-cultivation, the maximum values of 2.52 ± 0.41 mg/L and 2.40 ± 0.40 mg/L were achieved with *A.fumigatus* and *R.arrhizus* in a 1:1 ratio respectively. These were 2.73 and 2.6 times higher than pure cultivated alga. At the same time, the ratio of 2:1 recorded 1.52 ± 0.41 mg/L and 1.40 mg/L respectively. These values were 1.65 and 1.52 folds greater than control conditions. Currently, chlorophyll a has a vital role in photosynthesis by absorbing and transferring light energy [43].

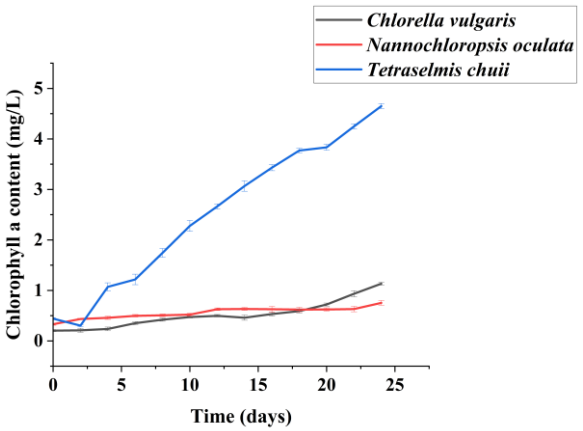

Figure 2. Growth characteristics expressed as chlorophyll a content (mg/L) of *Chlorella vulgaris*, *Nannochloropsis oculata*, and *Tetraselmis chuii*.

(Data are expressed as the means of three replicates and the standard deviation is indicated by error bars).

Besides, it has been consumed for health benefits as a nutraceutical agent with anti-inflammatory, antimutagenic, antimicrobial, and antioxidant properties [44]. The improved chlorophyll a content observed in microalgae grown at 1:1 and 2:1 algae/fungi ratio is possible because of light/shade adaptations that enhance the use of light energy. Certainly, microalgae chlorophyll a production can be manipulated by altering the culture conditions [45], which is not only cultivation mode-dependent but also species-specific [46]. On the other hand, the minimum values of chlorophyll a content were 1.40 mg/L and 1.11 ± 0.28 mg/L for *C.vulgaris* with *M.alpina* in ratios 1:1 and 2:1 respectively. In this case, the initial ratio for each co-partner is a main concern since it will affect the consortium outcome. It is interesting to note that the greatest chlorophyll a content can be reached only under the optimal dosage of the co-partners. Also, the content of chlorophyll a would actually decrease beyond this optimal dosage.

Cheirsilp and Torpee [47] reported that *Chlorella vulgaris* and *Aspergillus niger* co-cultivation led to a 23% increase in chlorophyll content compared to *C.vulgaris* grown alone. Also, Toscano et al. [48] found that the co-culture of *Chlorella sorokiniana* and *Trichoderma harzianum* resulted in a 32% higher chlorophyll content than the *Chlorella* monoculture.

A notable increase in the chlorophyll a content of *N.oculata* in co-culture with *A. fumigatus*, *R.arrhizus*, and *M.alpina* was observed (**Figure 3B**). chlorophyll a content of 1.56 ± 0.36 and 1.55 ± 0.35 mg/L were obtained once co-culturing with *M.alpina* in a 1:1 and 2:1 algal fungal ratio respectively. These were 2.52 and 2.5 folds higher than the Chlorophyll a content of *N.oculata* pure culture (0.62 ± 0.02 mg/L). For *N.oculata* with *A.fumigatus* in 1:1 and 2:1 algal fungal loading, chlorophyll a content reached 1.40 ± 0.21 and 1.37 ± 0.11 mg/L respectively. At this time, the ratios of 1:1 and 2:1 could be selected as the optimal ratios for achieving the maximum chlorophyll a content. The diversity of selected consortia has turned out to be the key factor for the chlorophyll an enhancement of algal species. The

selection criteria for successful co-cultivation must include specific co-partners adaptation for healthy growth and biomass composition. The change in chlorophyll a production is possibly due to some specific characteristics of co-partners involved in the consortia. Fungi can release nutrients into the culture medium, leading to algal growth promotion with chlorophyll a accumulation [49]. On the other hand, fungi can induce stress responses in algae, triggering the production of protective compounds like chlorophyll [50].

Xiong et al. [51] found that co-cultivating *Nannochloropsis oculata* with *Rhizopus oryzae* led to a 25% increase in chlorophyll content compared to the microalgae grown alone. In addition, Jiang et al. [52] reported a 22% increase in chlorophyll content of *Nannochloropsis oculata* when grown together with *Aspergillus niger*. Also, *Nannochloropsis oculata* and *Trichoderma harzianum* co-cultivation led to a 28% increase in chlorophyll content [24].

The lowest Chlorophyll a content of *N.oculata* in symbiosis with *R.arrhizus* was 1.17± 0.11 and 1.04 ± 0.34 mg/L for ratios of 1:1 and 2:1 respectively. These results signify that species selection is prime and one of the main criteria affecting the binary culture system. The pair selection may be mainly affected by communication profiling in a binary culture system. And/or screening from symbiotic interactions existing in nature.

The chlorophyll a content for *T.chuii* and the co-partners tested at two different ratios is shown in (**Figure 3C**). The greatest chlorophyll a content (8.16 ± 0.11 mg/L) was achieved after co-cultivation on the combination of *T.chuii* and *A.fumigatus* in a ratio of 2:1. Which was 1.94 times higher than chlorophyll a content of *T.chuii* individual culture (4.20 ± 0.20 mg/L). Additionally, in a ratio of 1:1 chlorophyll a content was 4.90 ± 0.85 mg/L which was 1.16 folds higher than that obtained in mono-cultured alga.

It is worth noting that applying microalgae and fungi co-cultivation in different ratios can affect the algal growth and chlorophyll a production. The results revealed that, in the co-cultivation of *T.chuii* with different fungi, much higher chlorophyll a content was obtained in a 2:1 ratio indicating that double alga/fungus loading can better create an appropriate growth condition for microalgae with high chlorophyll a accumulation. In symbiotic systems, algal cells assimilate inorganic carbon and generate biomass, which is consumed by fungi through heterotrophic metabolisms [53]. When the metabolic reactions are combined and complementary, this kind of co-culture can accumulate high chlorophyll content for desired processing [4]. Similarly, Muradov et al. [24] found that co-cultivating *Tetraselmis chuii* with *Morterella alpina* caused a 32% increase in chlorophyll content compared to the microalga grown alone. Also, Xiong et al. [51] observed a 29% increase in chlorophyll content when *Tetraselmis chuii* was co-cultivated with *Penicillium chrysogenum*.

The chlorophyll a content of *T.chuii* when co-cultivated with *R.arrhizus* was 6.43 ± 0.21 mg/L in a ratio of 2:1. It was 1.53 times higher than that of pure algal cultures (4.20 ± 0.20 mg/L). Concerning the 1:1 algal/fungal ratio, it is significant that chlorophyll a content declined to 4.12 mg/L after 24 hrs., then increased to 4.53 mg/L after 48 hrs. By the end of co-cultivation and after 72 hrs., it decreased to 4.20 mg/L, which was similar to that obtained before co-cultivation. At equal dosages of *T.chuii* and *R.arrhizus*, with an increase in culture time, the chlorophyll a content of microalgae first decreased then increased, and finally decreased during the co-culture. This means that, by extension in the co-culture period, the nutrients are not available and insufficient, consequently, the secreted metabolites by fungi act as substitute food for algal cells, causing increased chlorophyll a content. By this time, fungi may activate stress pathways, resulting in reduced chlorophyll a concentration.

The consortia of *T.chuii* and *M.alpina* has the lowest chlorophyll a content of 4.89 ± 0.17 and 4.71 ± 0.09 mg/L with ratios of 2:1 and 1:1 respectively. When *T.chuii* and *M.alpina* are co-cultivated, they form a dense culture, where the cells of fungi can physically shade the other microalgal species. *T.chuii* could not withstand these conditions. The shading effect can reduce the light availability for the microalgae [54], which may consequently lead to a decrease in chlorophyll production.

Considering results in Table 2, *C.vulgaris* showed the greatest biomass concentration after 72 hrs. co-cultivation with *A.fumigatus* at a ratio of 1:1. It reached 619.2 ± 0.97 mg/L, which was 2.83 folds higher than algal monoculture (218.5 mg/L). Moreover, it was 1.62 times greater than that obtained at a 2:1 ratio (383.2 ± 1.11 mg/L). *C.vulgaris* with *R.arrhizus* in a ratio of 1:1 achieved a biomass concentration of 570.1 ± 0.90 mg/L. That was 1.68 times higher than the biomass attained with a 2:1 algal/fungal ratio (339.83 ± 1.16 mg/L). *C.vulgaris* and *M.alpina* co-cultivation achieved biomass concentrations of 341.6 ± 0.46 and 272.7 ± 0.64 mg/L for 1:1 and 2:1 algal/fungal loading respectively. The fast-growing algae with plenty of biomass may correspond to factors such as specific fungal species and definite cell ratio. Optimizing these factors plays a role in providing the microalgal cells with sufficient energy and allowing further exposure to hyphae for benefit. Thus, it can potentially enhance growth and consequently affect biomass production. Similarly, when *Trichosporonoides spathulata* was co-cultivated with many species of *Chlorella* to study the advantage of the co-culture system, co-cultivation resulted in the overall highest biomass productivity [55, 56]. The co-culture yielded the greatest biomass of 12.2 g/L [55]. Also, Wrede et al. [25] found that *Chlorella reinhardtii* and *Chlorella vulgaris* co-cultivated with *Aspergillus fumigatus* resulted in biomasses of 0.54 and 0.47 g/L respectively.

Concerning *N.oculata* and *M.alpina* symbiosis Table 2, It was noted that the co-cultivation in ratios of 1:1 and 2:1 achieved approximately equal biomass of 453.3 and 453.0 mg/L respectively. They were found to be 2.52 folds higher than the biomass of the pure culture of *N.oculata* (179.7 ± 1.61 mg/L). At this point, for the best synergistic relationship, different algal/fungal ratios do not play an important role as they recorded the same biomass concentration. Actually, species specificity is the most dynamic aspect to be considered. *M.alpina* may be used to promote the growth of microalgal cells, help in consuming dissolved nutrients, and accordingly alter biomass composition.

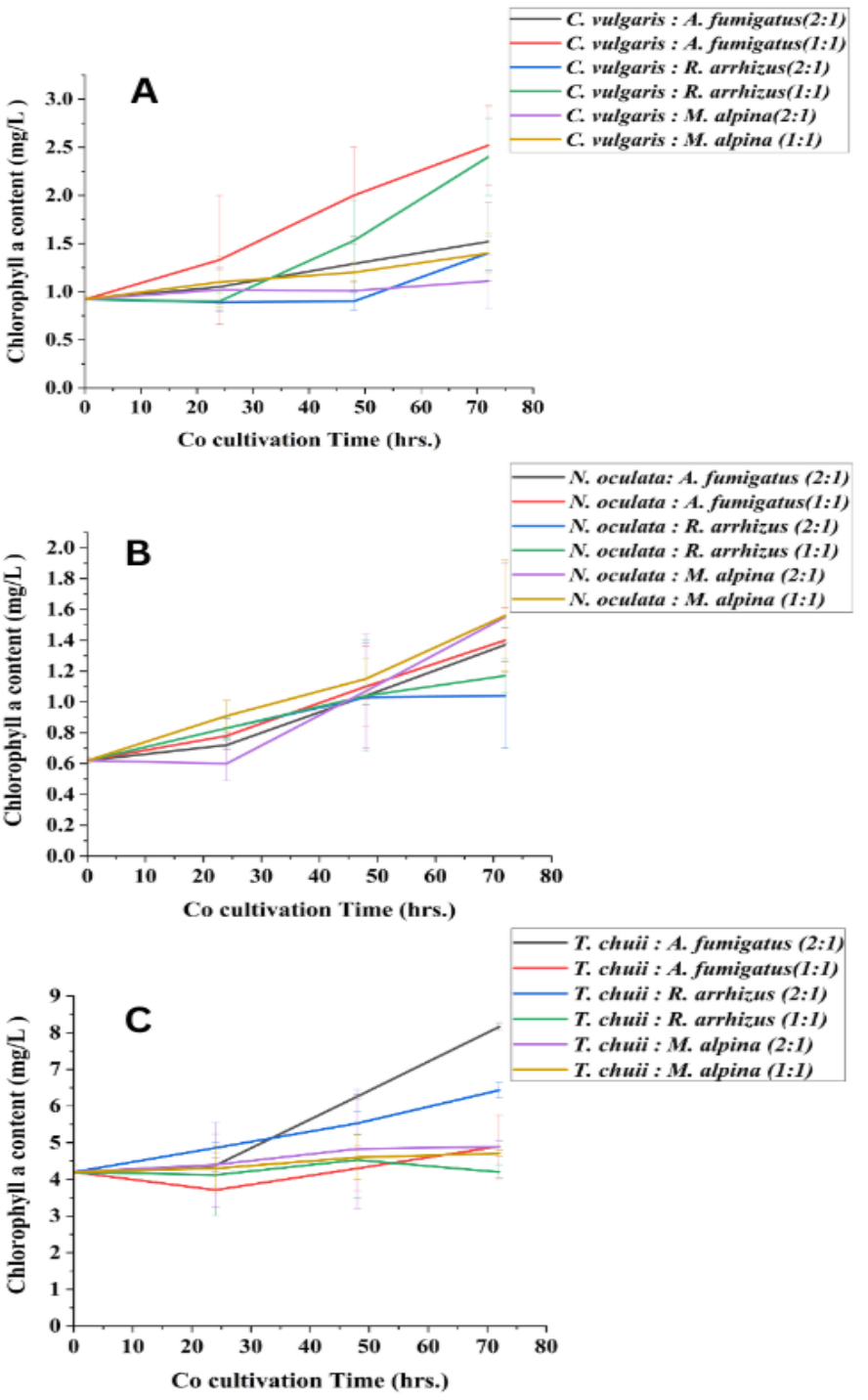

Figure 3: Chlorophyll a content(mg/L) of *Chlorella vulgaris* (A), *Nannochloropsis oculata* (B), and *Tetraselmis chuii* (C) during co-cultivation.

(Data are expressed as the means of three replicates and the standard deviation is indicated by error bars).

Table 2: Biomass concentration (mg/L) of *Chlorella vulgaris*, *Nannochloropsis oculata*, and *Tetraselmis chuii* co-cultured with *Aspergillus fumigatus*, *Rhizopus arrhizus*, and *Morterella alpina*

Algae	Fungi	Algae: Fungi ratio	Biomass concentration (mg/L)			
			Co-cultivation Time (hrs.)			
			0 hrs.	24 hrs.	48 hrs.	72 hrs.
C.vulgaris	*A.fumigatus*	2:1	$218.5^{p} \pm 1.15$	$228.5^{n} \pm 1.30$	$305.8^{h} \pm 1.15$	$383.2^{d} \pm 1.11$
		1:1	$218.5^{p} \pm 1.11$	$309.8^{g} \pm 0.53$	$464.5^{c} \pm 0.83$	$619.2^{a} \pm 0.97$
	R.arrhizus	2:1	$218.5^{p} \pm 0.51$	$219.9^{op} \pm 0.95$	$222.2^{o} \pm 0.80$	$339.83^{f} \pm 1.16$
		1:1	$218.5^{p} \pm 0.60$	$218.6^{p} \pm 1.17$	$368.3^{e} \pm 0.62$	$570.1^{b} \pm 0.90$
	M.alpina	2:1	$218.5^{p} \pm 1.18$	$231.4^{m} \pm 0.67$	$244.4^{l} \pm 0.59$	$272.7^{j} \pm 0.64$
		1:1	$218.5^{p} \pm 1.28$	$257.1^{k} \pm 0.80$	$296.2^{i} \pm 0.57$	$341.6^{f} \pm 0.46$
	p <0.001*					
N.oculata	*A.fumigatus*	2:1	$179.7^{k} \pm 1.61$	$208.6^{j} \pm 1.04$	$301.8^{f} \pm 1.61$	$395.0^{c} \pm 2.65$
		1:1	$179.7^{k} \pm 1.61$	$226.0^{i} \pm 2.0$	$317.3^{e} \pm 0.58$	$408.7^{b} \pm 0.58$
	R.arrhizus	2:1	$179.7^{k} \pm 1.61$	$239.5^{h} \pm 1.91$	$299.3^{f} \pm 1.53$	$301.5^{f} \pm 1.23$
		1:1	$179.7^{k} \pm 1.61$	$240.3^{h} \pm 6.81$	$300.7^{f} \pm 3.06$	$337.3^{d} \pm 2.52$
	M.alpina	2:1	$179.7^{k} \pm 1.61$	$173.7^{k} \pm 1.53$	$313.7^{e} \pm 3.06$	$453.0^{a} \pm 7.64$
		1:1	$179.7^{k} \pm 1.61$	$260.3^{g} \pm 8.50$	$333.3^{d} \pm 4.04$	$453.3^{a} \pm 3.0$
	p <0.001*					
T.chuii	*A.fumigatus*	2:1	$177.5^{hi} \pm 1.32$	$185.3^{g} \pm 1.53$	$264.7^{b} \pm 1.53$	$344.2^{a} \pm 1.26$
		1:1	$177.5^{hi} \pm 1.32$	$156.3^{k} \pm 4.04$	$181.7^{gh} \pm 3.06$	$207.6^{d} \pm 2.51$
	R.arrhizus	2:1	$177.5^{hi} \pm 1.32$	$205.8^{de} \pm 1.26$	$234.3^{c} \pm 1.53$	$271.3^{b} \pm 1.53$
		1:1	$177.5^{hi} \pm 1.32$	$174.0^{ij} \pm 4.58$	$169.0^{j} \pm 2.65$	177.4 ± 1.58
	M.alpina	2:1	$177.5^{hi} \pm 1.32$	$186.0^{g} \pm 2.65$	$202.8^{de} \pm 1.57$	$207.0^{d} \pm 3.46$
		1:1	$177.5^{hi} \pm 1.32$	$181.6^{gh} \pm 4.50$	$194.3^{f} \pm 2.52$	$198.5^{ef} \pm 2.29$
	p <0.001*					

The data shown are the average Biomass concentration from three biological replicates. Expressed using mean ± standard deviation.

Values in a column with different letters are significantly different according to the three-way analysis of variance with Post Hoc Test (Tukey) ($P < 0.05$), using SPSS version 25, IBM Corporation.

Biomass concentration of *N.oculata* and *A.fumigatus* in ratio 1:1 reached 408.7 ± 0.58 mg/L which was 13.7 mg/L higher than the biomass of 2:1 algal fungal ratio (395.0 ± 2.65 mg/L). *N.oculata* and *R.arrhizus* in a ratio of 2:1 recorded biomass concentration of 301.5 ± 1.23 mg/L. At the same time, a ratio of 1:1 recorded 337.3 ± 2.52 mg/L which was 1.87 times higher than that achieved in mono-cultured alga. Maintaining an equal proportion of alga and fungus can create a stable symbiotic environment for growing together. They may perhaps have harmonizing interactions in a consortium. Fungi can secrete enzymes that degrade complex organic matter, making it more accessible to alga for utilization and enhancing productivity in terms of biomass production [57]. Wrede et al. [26] found that *Nannochloropsis oculata* and *Aspergillus fumigatus* co-cultivation resulted in a biomass of 0.47±0.1g/L.

T.chuii co-cultured with *A.fumigatus*, *R.arrhizus*, and *M.alpina* showed a significant increase in total biomass concentration as observed in Table 2. The biomass yield of *T.chuii* before co-cultivation was 177.5 ± 1.32 mg/L. The final biomass concentration reached 207.6 ± 2.51 and 344.2 ± 1.26 mg/L for co-cultivation with *A.fumigatus* in ratios 1:1 and 2:1 respectively. Which was 1.17 and 1.94 times greater than that of mono-cultured *T.chuii*. Actually, *T.chuii* in the presence of *A.fumigatus*, potentially contributes to the accumulation of high total biomass. It persistently increased and reached its maximum whereas it is not in controlled cultures. Co-culture conditions can stimulate growth and promote nutrient assimilation, which encourages the growth of microalgal cells for possible biomass

production. In the symbiotic relationship between algae and fungi, the algal cells are capable of assimilating inorganic carbon, such as carbon dioxide, and using it to produce organic compounds and oxygen through photosynthesis. Consequently, fungi meet their requirement by utilizing organic compounds produced through algal photosynthesis [53]. Muradov et al. [24] confirmed that co-cultures of the marine microalga *Tetraselmis suecica* and fungi *Aspergillus fumigatus* showed maximum algal growth. Also, Wrede et al. [26] found that *Tetraselmis chuii* and *Aspergillus fumigatus* co-cultivation resulted in a biomass of 0.31±0.7 g/L.

The biomass concentration of symbiosis between *T.chuii* and *R.arrhizus* increased from 177.4 ± 1.58 mg/L with algal fungal loading of 1:1 until the maximum value was recorded in a ratio 2:1 (271.3 ± 1.53 mg/L). This double ratio plays a critical factor that can affect the whole performance of the co-cultivation process. The metabolites secreted by the fungi can act as additional food, resulting in a high rate of cell division causing denser cultures and microalgal biomasses. This verifies that *T.chuii* and *R.arrhizus* are co-dependent and possibly of interest. Chu et al. [4], Shokravi et al. [58] reported that *Tetraselmis suecica* and *Aspergillus fumigatus* co-culture leads to the improvement of microalgal growth.

Compared to *T.chuii* in symbiosis with *A.fumigatus* and *R.arrhizus*, it recorded the minimum biomass concentration with *M.alpina* in 2:1 and 1:1 ratio (207.0 ± 3.46 and 198.5 ±2.29 mg/L respectively). Such connections are not a positive one. This biological interaction is less effective and co-partners are not in harmony. Both species can regulate their metabolism during the cultivation period and satisfy their demands independently.

3.3. Morphological interactions of microalgae and fungi

The morphology of the studied organisms grown under controlled and co-cultured conditions (72hrs.), was observed by optical microscope (OPTIKA-Italy – Mi5200 Software). (**Figure 4**) shows the morphology of mono-cultured *C.vulgaris*, *N.oculata*, and *T.chuii*. The individual cells of *C.vulgaris* encompass microscopic unicellular spherical non-motile green cells with 2-10 µm diameter. And characterized by a thick cell wall containing a single cup-shaped chloroplast with pyrenoids. *N.oculata* appeared as spherical non-motile light green cells with a diameter of 2–5 µm with a smooth cell wall, a parietal chloroplast, and an eyespot that was always present. Cells of *T.chuii* typically appeared as dark green oval-shaped with a single large chloroplast containing one pyrenoid and a conspicuous eyespot with an average major diameter of 14.63 ± 0.574 µm and an average minor diameter of 9.591 ± 1.955 µm. With clear flagella located on one end of the cell.

Observing the structures of co-cultivated organisms under the light microscope (**Figure 5**) validated that the microalgal cells of *C.vulgaris*, *N.oculata*, and *T.chuii* were intertwining to mycelia of *A.fumigatus*, *R.arrhizus*, and *M.alpina*. And mutually tangled together to form the network morphology.

Certainly, the smooth fibrous nature of mycelia contributes to protecting algae cells. The structure of the microalgal cells is intact, which assists in reducing the release of algae cellular secretions to the surroundings. Consequently keeps the molecules and cell components without any loss. These results support the idea that filamentous fungi are capable of promoting the growth of microalgal cells. These results were in agreement with previous studies showing that microalgae, effectively utilize the fungi complex structure [59]. In addition, the interactions between functional groups of the cell surface possibly explained the co-cultivation mechanism [60]. Zheng et al. [61] reported that the structure and filamentous properties of the fungal hyphae provided more sites and a large specific surface area for the microalgae interaction. Wang et al. [60] observed the structure of *Chlorella vulgaris-Aspergillus niger* co-pellets and found a large number of *Chlorella vulgaris* cells form a network with fungal hyphae, which support effective absorption.

Conclusion

This study validates the possibility of microalgae and fungi co-cultivation. Organisms selected for co-cultivation showed significant potential for enhancement of algal biomass to overcome the major challenges facing microalgal commercialization. The data suggest that by screening different algae and fungi strains, adjusting the algal-fungal loading, and varying the co-cultivation time, microalgal biomass production can be tailored and optimized. This could be attributed to various factors such as interactions between the selected organisms, nutrient availability, and metabolic pathways.

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Authors' Contribution

Conceptualization N.M., methodology, D.O., A.W., E.F., G.Y.; validation, N.M., E.F., G.Y., A.W., D.O.; writing original draft preparation, E.F., D.O.; writing review and editing, N.M., E.F., D.O. All authors have read and agreed to the published version of the manuscript.

Data Availability:

All the original data are available upon reasonable request from correspondence authors.

Conflict of Interest

The authors declare no conflict of interest.

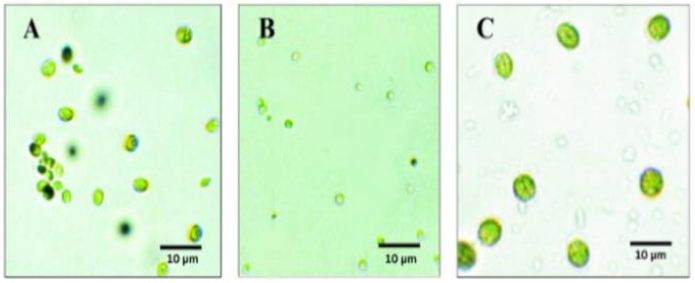

Figure 4. light Micrographs of (a) *Chlorella vulgaris*, (b) *Nannochloropsis oculata* and (c) *Tetraselmis chuii*.

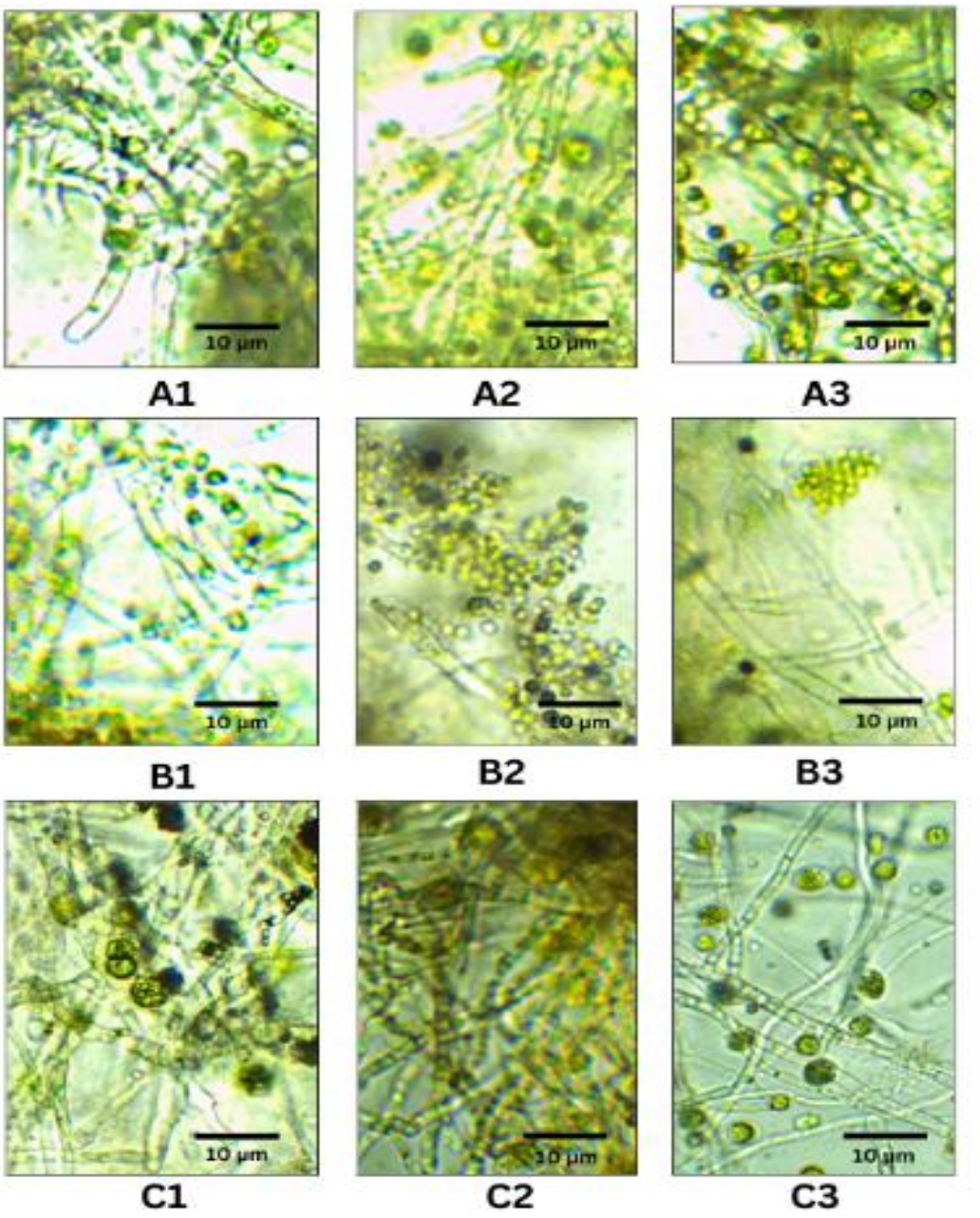

Figure 5. Light microscopic images of microalgal fungal interactions.

Chlorella vulgaris microalgae cells attached to *Aspergillus fumigatus* (A1), *Rhizopus arrhizus* (A2), and *Morterella alpina* (A3).

Nannochloropsis oculata microalgae cells attached to *Aspergillus fumigatus* (B1), *Rhizopus arrhizus* (B2), and *Morterella alpina* (B3).

Tetraselmis chuii microalgae cells attached to *Aspergillus fumigatus* (C1), *Rhizopus arrhizus* (C2), and *Morterella alpina* (C3).

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