



Bioflocculation of *Euglena gracilis* via direct application of fungal filaments: a rapid harvesting method

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Abstract

The high cost and environmental impact of traditional microalgal harvesting methods limit commercialization of microalgal biomass. Fungal bioflocculation of microalgae is a promising low-cost, eco-friendly method but the range of fungal and microalgal species tested to date is narrow. Here, eight non-pathogenic, filamentous fungi were screened for their ability to self-pelletize and flocculate *Euglena gracilis* (ca. 50 µm motile microalga) in suspension. Self-pelletization was tested under various rotational speeds, and species which formed pellets ($\varnothing > 0.5$ cm) were selected for harvesting tests. Filaments of each species were combined with *E. gracilis* at various ratios based on dry weight. Harvesting efficiency was determined by measuring the change in cell counts over time, and settling of the flocs was evaluated by batch settling tests. Three fungal species, *Ganoderma lucidum*, *Pleurotus ostreatus*, and *Penicillium restrictum*, were able to reliably flocculate and harvest 62–75% of the microalgae while leaving it unharmed. The results demonstrated that self-pelletization, harvesting, and settling were dependent on the fungal species. The fungi to algae ratio also had significant but contrasting effects on harvesting and settling. In balancing the needs to both harvest and settle the biomass, the optimal fungi to algae ratio was 1:2. The application of fungal filaments to microalgae in suspension produced readily settling flocs and was less time-consuming than other commonly used methods. This method is especially attractive for harvesting microalgal biomass for low-value products where speed, low cost, and cell integrity is vital.

Keywords Fungal pelletization · Bioflocculation · Microalgal harvesting · Filamentous fungi · *Euglena gracilis*

Introduction

Microalgal biomass is a prominent source for a wide variety of high-value products such as food and beverages, supplements, cosmetics, and nutraceuticals (Biorizon Biotech 2018). The global market of microalgae-based products in 2019 was 1.8 billion US\$ and is projected to be 4.2 billion US\$ by 2031 (Transparency Market Research 2021). However, the high production costs (> 5 EUR kg⁻¹) make commercialization of lower value products such as biofuel and feed unfeasible (Mathimani and Mallick 2018; Muhammad

et al. 2021). For example, for algal-based biofuels to be competitive with crop-based biofuels, the production costs of microalgal biomass should be < 1 EUR kg⁻¹ (Biorizon Biotech 2018). Microalgal harvesting accounts for as much as 20–30% of the production costs and has a high environmental impact (Fasaei et al. 2018; Li et al. 2020; Muhammad et al. 2021). Reducing the costs and energy demand of algal harvesting would be a step towards producing microalgal biomass more economically and sustainably.

Traditional microalgal harvesting methods fall into one of three categories of separation methods: mechanical, physical, or chemical (Barros et al. 2015; Fasaei et al. 2018). The most efficient of these methods are the mechanical separation methods of centrifugation and filtration which can harvest up to 99% of the microalgae. However, these methods have high operating costs (Barros et al. 2015; Fasaei et al. 2018; Najjar and Abu-Shamleh 2020). The commonly used physical methods, flotation and sedimentation, are not as efficient as centrifugation or filtration so are usually combined with chemical flocculation employing metal salts or

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polymers to increase the size of the flocs for improved harvesting efficiency ($\geq 99\%$) (Mata et al. 2010; Uduman et al. 2010; Barros et al. 2015). However, flotation is expensive due to its high energy consumption added to the cost of flocculants (Uduman et al. 2010). Flocculation followed by sedimentation is inexpensive compared to other separation methods as there is little to no energy requirement, no specialized equipment needed, and inorganic flocculants are cheap (Muhammad et al. 2021). However, metal salts carry environmental costs (Chen et al. 2011) and contaminate the biomass limiting its downstream processing and applications (Singh and Patidar 2018). Polymers may be inorganic or organic, with organic polymers presenting a more environmentally friendly alternative (Vasistha et al. 2021). However, organic flocculants tend to be expensive (Barros et al. 2015), and do not flocculate some algal species well (Mathimani and Mallick 2018).

Bioflocculation holds promise as a cheap and eco-friendly alternative. It possesses the general benefits of flocculation (low energy consumption, no specialized equipment needed) while substituting inorganic flocculants with biodegradable ones. It also allows recycling of the growth medium further reducing production costs (Chen et al. 2018; Yin et al. 2020). The simplest form of bioflocculation is autoflocculation of microalgae, i.e., flocculation is induced by the microalgae themselves (Spilling et al. 2011; Muhammad et al. 2021). However, not all microalgal species can autoflocculate (Spilling et al. 2011); its induction may require non-ideal conditions reducing growth and lipid content (Pahl et al. 2013; Magdouli et al. 2016); it may incur chemical costs (Besson and Guiraud 2013) and can be unreliable (Ummalyma et al. 2017). Different bioflocculating agents such as “sticky” extracellular polymers (EPS), bacteria, and fungi can also be used for aggregating microalgal cells (Alam et al. 2016). EPS are naturally produced by many organisms (Mishra and Jha 2009), but the industrial production and use of EPS is unfeasible since extraction and purification methods are complex and expensive (Pahl et al. 2013), and their efficiency unpredictable (Li and Yang 2007). Bacterial flocculants give reliable and high harvesting efficiencies (Ummalyma et al. 2017; Li et al. 2020), and in the case of oleaginous species, it can also increase lipid yields (Chen et al. 2011; Salim et al. 2011). However, additional organic carbon is required for the bacteria to grow and harvest the algae, which increases costs and might also encourage growth of undesirable bacterial strains (Lee et al. 2009; Salim et al. 2011).

Like bacteria, fungal bioflocculants give reliable and high microalgal harvesting efficiencies (Bhattacharya et al. 2017; Ummalyma et al. 2017). Fungi employ, often simultaneously, several different microalgal harvesting mechanisms such as self-pelletization, excretion of EPS, attraction, entrapment (Egede et al. 2016; Magdouli et al. 2016),

and attachment to the fungal cell wall (Wrede et al. 2014; Du et al. 2018). However, fungi are more attractive bioflocculants than bacteria because of their higher harvesting efficiencies (Zhou et al. 2012; Li et al. 2020; Nazari et al. 2020), greater potential to improve total biomass yields and value due to their nutrient, enzyme, and biochemical content (Egede et al. 2016; Li et al. 2020), and larger, denser flocs (Nguyen et al. 2019; Zhao et al. 2019; Kim et al. 2020; Pei et al. 2021). Bacteria-microalgae flocs tend to be < 5 mm and less dense (Lee et al. 2013; Nguyen et al. 2019; Kim et al. 2020; Pei et al. 2021), while fungi-microalgae flocs have more densely packed structure and may grow to be > 5 mm (Zhao et al. 2019; Kim et al. 2020) improving settling and dewatering of the biomass (Benjamin and Lawler 2013). As fungi can potentially add to the nutrient and biochemical value of the total biomass, there is no need to remove the fungal biomass after harvesting and therefore no contamination issues unlike bacteria (Egede et al. 2016; Chu et al. 2021a). However, to be a suitable harvesting alternative, the fungi used should be non-pathogenic, should be safe for downstream applications (e.g., feed and fertilizer), and should not disrupt the microalgal cell structure (Ummalyma et al. 2017).

Previous studies applying fungi to microalgal harvesting have focused on harvesting small, non-motile microalgae such as *Chlorella* spp. using mainly *Aspergillus* spp. (Barros et al. 2015; Li, et al. 2020), many of which are pathogenic. However, the wider applicability of fungal harvesting of microalgae should also be evaluated for larger, motile microalgal species of commercial interest such as *Euglena gracilis* (30–50 μm) (Gissibl et al. 2019) and for other bioflocculating fungal species. Here, a novel and rapid microalgal harvesting method was developed, directly applying fungal filaments to *E. gracilis* cultures to enable immediate bioflocculation and harvesting, cutting out many of the preparation steps needed in pellet- and spore-assisted methods (Zhang and Hu 2012; Al-Hothaly et al. 2015; Mackay et al. 2015). The method was tested using eight filamentous fungal species (*Ganoderma lucidum*, *Geotrichum candidum*, *Lentinus tigrinus*, *Paecilomyces variotii*, *Penicillium corylophilum*, *Penicillium restrictum*, *Pleurotus ostreatus*, and *Trametes versicolor*) which are non-pathogenic (COGEM 2021), safe for use in feed and fertilizer (Hindumathi and Reddy 2011; Adams et al. 2019), produce industrially important biochemicals and enzymes (Bishop et al. 2015; Purchase 2016; Yadav et al. 2019), and survive in polluted/toxic environments (Purchase 2016), making them excellent candidates for production of high-value biomass (Purchase 2016; Yadav et al. 2019). The aims of this study were (1) to evaluate the ability of the filaments of the eight fungal species to self-pelletize and flocculate *E. gracilis* in suspension, (2) to evaluate the harvesting and dewatering ability of the resultant flocs via batch settling tests, and (3) to determine the

optimal fungi to algae ratio needed for maximal harvesting efficiency and settling.

Materials and methods

Organisms and culture methods

Eight filamentous fungal strains were used in this study: *Ganoderma lucidum* (NRRL 66,208), *Geotrichum candidum* (NRRL Y-552), *Lentinus tigrinus* (HAMBI FBCC 645), *Paecilomyces variotii* (NRRL 1115), *Penicillium corylophilum* (NRRL 802), *Penicillium restrictum* (NRRL 3381), *Pleurotus ostreatus* (NRRL 3526), and *Trametes versicolor* (NRRL 66313). Each strain was transferred to Potato Dextrose Agar (PDA) plates and incubated at 24 °C for one week, transferred to 4 °C and refreshed every 3–4 months. Liquid suspensions were made by cutting ~2 cm² from the outer edge of the fungal mass, suspending in 40 mL of Yeast Malt Broth (YMB) (NRRL Medium No. 6 w/o agar) in 250 mL culture flasks and incubating at 24 °C until they were in the exponential growth phase (approx. 2 to 4 weeks depending on the species). Liquid suspensions were then maintained in YMB for 1–2 months depending on the growth rate of the species. *P. ostreatus* was chosen as the reference organism for microalgal harvesting method development due to its fast growth rate (Egede et al. 2016; Bellettini et al. 2019), and proven ability to form pellets and harvest algae (Luo et al. 2019). The microalgal strain, *Euglena gracilis* (CCAP 1224/5Z), was maintained in Modified Acid Medium (MAM) (Olaueson and Stokes 1989), with *E. gracilis* Medium (EG) (CCAP) at a 1:4 (EG:MAM) ratio at 19 °C under a light intensity of 30–40 μmol m⁻² s⁻¹ with a light:dark cycle of 14:10.

Fungal self-pelletization

The flocculation potential of the eight species was investigated by testing their ability to self-pelletize under rotation on a horizontal shaker. Though self-pelletization can be induced in several ways, depending on the species (Liu et al. 2007), agitation is an easy method of inducing pellet formation in several filamentous fungi (Gultom et al. 2014; Chen et al. 2018; Luo et al. 2019). General pelletization ability of all fungi was first tested in a total volume of 100 mL and agitated at 110 RPM for approximately 1 week. Species were chosen for further study based on how well they pelleted (rapid, well defined, >0.5 cm Ø). In the case of the two *Penicillium* spp., the faster-growing species was chosen for further study. The effect of various rotational speeds (50, 80, 90, 100, 110, 120, and 150 rpm) and total culture volumes (50 and 100 mL) on self-pelletization and floc formation was then tested using *P. ostreatus* as the test organism. All samples were inoculated

with a 10% v/v liquid fungal suspension and incubated on a horizontal shaker at room temperature (RT), i.e., 23 °C. The pH of the fungal cultures was measured at the start of the tests and at the end using Fisherbrand pH Fix 2.0–9.0 pH strips. Conditions resulting in optimal floc formation were used for all subsequent experiments.

Sampling and measurement protocols of harvesting experiments

Fungal harvesting of *E. gracilis* was optimized in a series of experiments investigating the effect of fungi to algae ratio and time on harvesting efficiency and settling properties. Before each experiment, the dry weights (g DW L⁻¹) of the fungal and algal cultures were determined by filtering 2–5 mL samples onto pre-combusted, pre-weighed Whatman GF/F filters in triplicate, drying the filters at 60 °C overnight, and weighing them again. Dry weight was calculated using the following equation:

$$\frac{W_2 - W_1}{V} \quad (1)$$

where W_1 is the weight (g) of the filters prior to addition of the sample, W_2 is their weight after drying, and V is the volume of sample filtered (L).

Inoculum concentrations of the fungal suspensions were diluted to the same g DW L⁻¹ concentration (Eq. 1) as the *E. gracilis* culture. Fungi and algae cultures were then combined in the given ratios for a total volume of 50 mL in 250 mL Erlenmeyer flasks and incubated at 90 rpm, under ambient laboratory light conditions. Each test was set up in replicates of four and the pH was checked prior to combining the fungi and algae in the various ratios and immediately upon combination. The efficiency of photosystem II (the ratio of variable to maximum fluorescence, F_v/F_m) was measured with AquaPen-C AP 110-C fluorometer and aliquots for *E. gracilis* cell enumeration were taken at various time points during the experiments. Aliquots were preserved with acid Lugol's solution and stored in glass vials at 4 °C until counted with a FlowCam macrofluid imaging instrument (Yokogawa Fluid Imaging Technologies Inc., USA) at 10× magnification. So as not to clog the 100 μm flow cell used, large fungal particles were removed by filtration (80 μm filter). Cell count data was converted to harvesting efficiencies as percentages based on the starting concentrations using the following equation:

$$\frac{C_1 - C_2}{C_1} * 100 \quad (2)$$

where C_1 is the starting concentration (cells mL⁻¹) and C_2 the final concentration of *E. gracilis* cells.

To determine how readily the resultant flocs settled, batch settling tests were carried out at the end of each harvesting experiment. This was done by gently resuspending the flocs, combining the four replicates into one flask, and gently pouring into a 250 mL glass measuring cylinder. The changing height of the floc-liquid interface was recorded at various time points. In phase 1, the height of the interface was measured at 0, 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 30, 45, and 60 min. Based on the results of this batch settling test, the height of the interface was only measured at 0-, 5-, 10-, 15-, 20-, and 30-min intervals in subsequent phases 2 and 3. The data from each experiment were then plotted to produce a batch settling curve.

Experimental phases

Phase 1 To fine-tune settling time and the optimal fungi to algae ratio for maximal harvesting efficiency, four different fungi (*P. ostreatus*) to algae (*E. gracilis*) ratios (1:0.5, 1:1, 1:2, 1:3), on a dry weight basis, were investigated. The starting cell count of the *E. gracilis* culture was 300,000–350,000 cells mL⁻¹ (0.66 g L⁻¹). The *E. gracilis* stock culture was sampled for cell counts and F_v/F_m , as were the suspensions of each the experimental replicates over time (immediately upon addition of fungi, at 6 h, every other hour from 6 to 10 h, and every 4 h from 10 to 18 h).

Phase 2 Based on the harvesting and settling results from phase 1, the experiment was narrowed to two ratios of *P. ostreatus* to *E. gracilis* (1:1 and 1:2) over a 6-h period. This time, the experiments were conducted at a higher algal concentration of 400,000–450,000 cells mL⁻¹ (0.89 g L⁻¹), and samples were taken just before the addition of fungi, immediately upon addition of fungi, and hourly thereafter for up to 6 h.

Phase 3 The harvesting and settling results from phase 2 led to further narrowing to a ratio of 1:2 (fungi:algae) over a 4-h period. The harvesting ability of four other strains, *G. lucidum*, *P. variotii*, *P. restrictum*, and *T. versicolor*, was

then tested at a ratio of 1:2 over 4 h, with 400,000–450,000 cells mL⁻¹ (~0.56 g L⁻¹) *E. gracilis* starting concentration. The suspensions were sampled just before the addition of fungi, immediately upon addition of fungi and every half hour for up to 4 h.

Statistical analysis

The differences in harvesting efficiencies (see Eq. 2) between the two *P. ostreatus* to *E. gracilis* ratios and fungal species were tested with Student's *t*-test and one-way ANOVA together with Tukey's post hoc tests, respectively, at the time points where maximal harvesting occurred (T_{max}). Normality of distribution was tested with Shapiro–Wilk's test and the homogeneity of variances with Levene's test in both cases. All the procedures were conducted using SPSS version 26.

Results

Self-pelletization and floc formation

Six of the eight fungal species formed pellets. Pellet formation began within 1–2 days, depending on the species, and most were well-formed and abundant within 5–7 days. For most of the species, the diameter of the pellets was in the range of 1–3 cm (Table 1, Online resource 1). Based on visual observations, it was clear that increased rotational speeds led to increasing numbers of pellets at decreasing sizes while increased volume had the opposite effect. Also, the pellets formed at high rotational speed appeared visibly smoother and denser than pellets formed at lower speeds which were fuzzy and less dense in appearance (Online resource 2). A culture volume of 50 mL and rotational speed of 90 rpm was found to be optimal for self-pelletization and floc formation, i.e., rough, loose, defined spheres > 0.5 cm ϕ .

Table 1 Ability of eight fungal species to form pellets at 110 rpm at room temperature over a week's time (+ denotes pellet formation, – denotes no pellet formation). Pellet diameter (cm) and pH measurements of the cultures at the beginning and end of the period are also given for each species

Species	Starting pH	pH at end	Pellet formation	Diameter (cm)
<i>Geotrichum candidum</i>	5.5	7.0	-	NA
<i>Ganoderma lucidum</i>	5.5	5.0	+	0.5–1
<i>Lentinus tigrinus</i>	5.5	5.5	-	NA
<i>Paecilomyces variotii</i>	5.5	5.5	+	> 4
<i>Penicillium corylophilum</i>	6.5	NA	+	1–2
<i>Penicillium restrictum</i>	6.0	5.0	+	0.5–4
<i>Pleurotus ostreatus</i>	4.0	4.0	+	0.5–3
<i>Trametes versicolor</i>	5.5	5.0	+	> 4

Phase 1. Harvesting of *E. gracilis* with *P. ostreatus* at four ratios

The harvesting efficiency of *E. gracilis* was highest at a fungi to algae ratio of 1:0.5 and lowest at 1:3. Maximum harvesting took place within ~6 h after which the cell count fluctuated until it began to rise again after 10 h (Fig. 1a). Maximum settling of flocs occurred mostly within the first 5 min for all ratios. However, flocs from the 1:2 and 1:3 ratios settled fastest and most compactly (Fig. 1b, Online resource 3), while biomass from the 1:0.5 and 1:1 ratios settled very little or not at all (Fig. 1b, Table 2).

Phase 2. Harvesting of *E. gracilis* with *P. ostreatus* at two ratios

Attraction of algae to the fungi began immediately upon addition of the fungus, and within an hour, green aggregates had formed at both ratios. At the 1:1 ratio, a maximum harvesting efficiency of $70\% \pm 2.9\%$ SE was reached

within 2 h, while at the 1:2 ratio, T_{max} was reached an hour later with a mean harvesting efficiency of $64\% \pm 1.7\%$ SE. However, there was no significant difference in the maximum harvesting efficiency between the two ratios (Student's *t*-test, $t(6) = 1.633, p = 0.15$). After T_{max} , *E. gracilis* cell count rose slightly at both ratios (Fig. 2a). Flocs from the 1:2 ratio settled more quickly and compactly than those from the 1:1 ratio (Fig. 2b), with a marked difference in settling rates within the first 5 min. After 5 min, little settling was observed at either ratio and settling rates were similar (Table 2).

Phase 3. Harvesting of *E. gracilis* with other filamentous fungal species

As in phase 2, attraction of algae to the fungi was observed immediately upon addition of *G. lucidum*, *P. ostreatus*, *P. restrictum*, and *T. versicolor* whereas no floc formation was observed for *P. variotii*. Flocculation time varied between species, with *G. lucidum* and *T. versicolor* flocculating

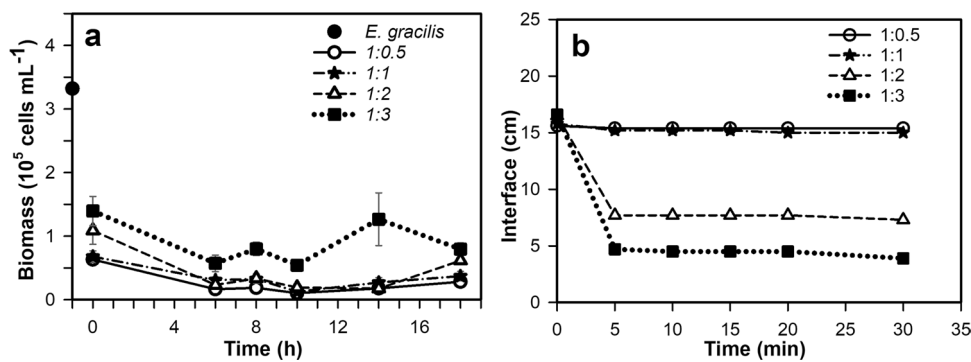


Fig. 1 Harvesting of *Euglena gracilis* by *Pleurotus ostreatus* at four different fungi to algae ratios, i.e., 1:0.5, 1:1, 1:2, and 1:3. **a** *E. gracilis* cell counts in the suspension at each fungi to algae ratio (mean \pm SE, $n = 4$) over time. Cell counts are shown for the following time points: *E. gracilis* stock culture (T-1), immediately upon addition of fungi to each replicate (T_0), every other hour from 6–10 h, and every 4 h thereafter. **b** Batch settling curves showing the change in height of the floc/liquid interface over time. Height of the interface at regular intervals for the first 30 min is given

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Table 2 Comparison of the extent and rate of settling of the fungi/algae flocs from all harvesting experiments (phases 1–3) within 30 min

Experiment	Ratio (fungi:algae)	Height of interface (cm)			Settling rate (cm min ⁻¹)	
		0 min	5 min	30 min	0–5 min	5–30 min
<i>P. ostreatus</i> / <i>E. gracilis</i> at four ratios	1:0.5	15.6	15.4	15.4	0.20	0.00
	1:1	15.8	15.2	15.0	0.60	0.20
	1:2	16.5	7.7	7.3	8.80	0.40
	1:3	16.6	4.7	3.9	11.90	0.80
<i>P. ostreatus</i> / <i>E. gracilis</i> at two ratios	1:1	16.9	7.0	6.5	1.98	0.02
	1:2	16.7	3.9	3.5	2.56	0.02
<i>G. lucidum</i>	1:2	16.1	3.2	3.0	2.58	0.04
<i>P. ostreatus</i>	1:2	16.7	3.9	3.5	2.56	0.08
<i>P. restrictum</i>	1:2	16.3	2.3	1.5	2.80	0.16

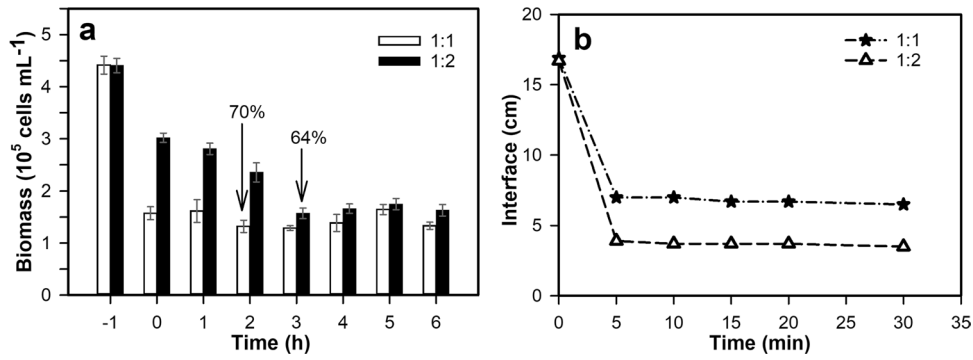


Fig. 2 Harvesting of *Euglena gracilis* by *Pleurotus ostreatus* at two fungi to algae ratios, i.e., 1:1 and 1:2. **a** *E. gracilis* cell counts in the suspension at both ratios (mean ± SE, n=4) over time. Cell counts are shown for the following time points: before addition of fungi (T-1),

immediately upon addition of fungi (T0) and hourly for up to 6 h. Harvesting efficiency at T_{max} is indicated by arrows. **b** Batch settling curves showing the change in height of the floc/liquid interface over 30 min

Table 3 The extent and speed of flocculation and the harvesting efficiency of *Euglena gracilis* by each fungal species (during phases 2–3). The time at which green aggregates began to form (floc formation starting time), time when T_{max} was reached, and the harvesting efficiency of *E. gracilis* for each fungal species is indicated

Fungal species	Ratio (fungi:algae)	Approx. floc formation starting time (min)	T_{max} (h)	Harvesting efficiency
<i>P. ostreatus</i>	1:1	45–60	2	70%
<i>P. ostreatus</i>	1:2	45–60	3	64%
<i>T. versicolor</i>	1:2	15–30	NA	NA
<i>G. lucidum</i>	1:2	20–35	1	62%
<i>P. restrictum</i>	1:2	~60	2	75%

first (Table 3). However, *T. versicolor* flocs fell apart after 1.5–2 h.

For all three flocculating fungal species, maximum harvesting efficiency was reached within 3 h. Maximum

harvesting efficiency was significantly higher for *P. restrictum* ($75\% \pm 0.8$ SE) than for *P. ostreatus* ($65\% \pm 1.7$ SE) and *G. lucidum* ($62\% \pm 1.5$ SE) (one-way ANOVA, $F(2,9) = 23.926$, $p < 0.001$, Tukey post hoc, $p = 0.001$), which performed similarly (Tukey post hoc, $p < 0.001$). After T_{max} , the *E. gracilis* cell count rose slightly in all three cases (Fig. 3a). Though flocculation starting times and T_{max} varied between species, species that formed flocs and arrived at T_{max} faster did not result in higher harvesting efficiencies (Table 3).

The flocs from all flocculating fungal species settled within the first 5 min. However, the flocs settled faster and more compactly when *E. gracilis* was harvested with *P. restrictum* than when harvested with *G. lucidum* or *P. ostreatus* (Fig. 3b). After 5 min, the settling rates of *G. lucidum* and *P. ostreatus* flocs were negligible but *P. restrictum* flocs continued to settle at a slow rate (Fig. 3b, Table 2).

All experiments resulted in loose, non-defined flocs that were more of a continuous aggregate rather than distinct

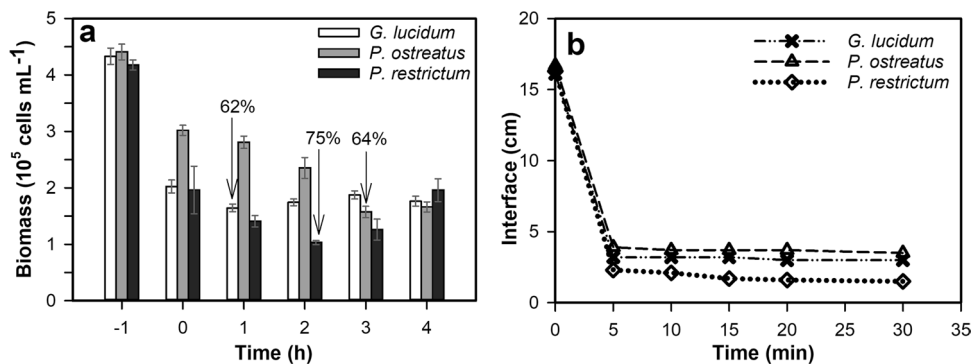


Fig. 3 Harvesting of *Euglena gracilis* by three different fungal species at a 1:2 (fungi:algae) ratio. **a** *E. gracilis* cell counts in the suspension (mean ± SE, n=4) over time. Cell counts are shown for the following time points: before addition of fungi (T-1), immediately upon

addition of fungi (T0), and hourly for up to 4 h. Harvesting efficiency at T_{max} is indicated by arrows. **b** Batch settling curves showing the change in height of the floc/liquid interface over 30 min

pellets. However, based on visual observations, the aggregate density was shown to vary by ratio and species and the density hierarchy was found to be 1:3 > 1:2 > 1:1 > 1:0.5 and *P. ostreatus* > *P. restrictum* > *G. lucidum*, respectively (Online resource 3, 4).

The pH and photochemical efficiency of the cultures

The pH range of the pelletization fungal cultures was 4.0–6.5 just after inoculation and 4.0–7.0 after 1 week of rotation (Table 1). The pH of the *E. gracilis* culture used for all experiments was pH 7.0. Upon addition of *G. lucidum*, *P. variotii*, and *T. versicolor* to the algae, the pH dropped from 7.0 to 6.5, while addition of *P. ostreatus* caused an even larger pH drop (3.5/4.0). Addition of *P. restrictum* on the other hand resulted in a slight pH increase (pH 7.5) (Table 4). The photochemical efficiency (F_v/F_m) of the microalgae was not affected by the addition of fungi and remained stable around 0.5 throughout all experiments.

Discussion

Pelletization

There were clear species-specific differences in self-pelletization and not all the fungal species screened were able to self-pelletize under rotation. Also, pellet size and shape differed between the species. Similar observations have also been made in earlier studies. Al-Hothary et al. (2015) found the ability to form pellets under rotation varied by fungal species, and Muradov et al. (2015) found that the pelletizing species produced varying numbers of pellets and morphologies. This is probably due to the species specificity nature of self-pelletization via rotation (Alam et al. 2016). Another reason may be the difference in the strength and number of

hydrophobic proteins (hydrophobins)—thought to be integral to self-pelletization—from species to species (Zhang and Zhang 2016).

Culture volume and rotational speed had a marked effect on pellet formation and morphology. The self-pelletizing tests using *P. ostreatus* showed that rpm correlated directly to pellet number and density but inversely to pellet size and surface roughness. The influence of increased agitation on pellet morphology resulting in smaller pellets has been well documented (Veiter et al. 2018; Luo et al. 2019; Zhao et al. 2019). Agitation also affects pellet morphology. For instance, Luo et al. (2019) found that at 100 rpm loose, rough, mid-sized pellets were formed while an increase of rpm to 150 caused high energy dissipation resulting in a larger number of small, dense, smooth pellets. Cui et al. (1997) suggested that increased shear forces chip off hyphae from the outer pellet limiting pellet size and that the hyphal fragments reseed new pellets increasing pellet number. Volume was negatively correlated to pellet number but positively correlated to pellet size. This is probably due to the decrease in turbulence which was observed in our study when culture volume was increased. As turbulence increases so too does energy dissipation (Zhou 1997), increasing shear forces on the filaments (Cui et al. 1997). Conversely, a decrease in turbulence will result in less shear force allowing pellets to grow larger. It stands to reason that an increase in volume coupled with a decrease in rotational speed would both reduce turbulence and shear forces until at some point energy dissipation would be insufficient for agitation of the biomass. This could explain why no pellets were formed when pelletization trials were conducted in a total volume of 100 mL at < 90 rpm.

The pH of the pelletization tests remained generally the same throughout the pelletization tests, except for *G. candidum* (pH increased) and *P. restrictum* (pH decreased). The pH of the fungal culture is an important factor in pellet formation as pH drives the electrostatic and hydrophobic interactions involved in pellet formation (Veiter et al. 2018). Acidic pH generally favors pellet formation due to changes in the functional groups on the surface of hyphae making them more hydrophobic (Veiter et al. 2018; Chu et al. 2021a). Changes in pH can change the surface properties of fungi which then influences pellet formation and morphology. Moreover, species are affected differently by pH (Metz and Kossen 1977), with pellet formation occurring at different pH (Liu et al. 2007; Prajapati et al. 2014). For the non-pelletizing species, pH possibly played a role in the case of *G. candidum* but not *L. tigrinus*, which did not form pellets even though pH remained acidic. *G. candidum* is a yeast which has retained many filamentous fungal genes and their resulting features after the evolutionary filamentous fungi-yeast split (Morel et al. 2015). Despite this retention of some filamentous fungal genes and their filamentous appearance

Table 4 pH measurements of all *E. gracilis* harvesting experiments (phases 1–3) immediately upon addition of fungi to algae

Experiment	Ratio (fungi:algae)	pH
<i>P. ostreatus</i> / <i>E. gracilis</i>	1:0.5	3.5
	1:1	3.5
	1:2	4
	1:3	4
<i>P. ostreatus</i> / <i>E. gracilis</i>	1:1	3.5
	1:2	3.5
<i>G. lucidum</i> / <i>E. gracilis</i>	1:2	6.5
<i>P. restrictum</i> / <i>E. gracilis</i>	1:2	7.5
<i>P. variotii</i> / <i>E. gracilis</i>	1:2	6.5
<i>T. versicolor</i> / <i>E. gracilis</i>	1:2	6.5

to the naked eye, it may be that unlike true filamentous fungi they are unable to form pellets. This, rather than any other factor, is probably the main reason for the lack of pellet formation in this species. Inoculum concentration, temperature, and media composition have also been found to affect pellet formation and morphology (Prajapati et al. 2014; Veiter et al. 2018) but were not investigated in this study.

Flocculation

Self-pelletization is a pre-requisite for harvesting, but not all self-pelletizing species were able to form flocs with the algae. Out of the six self-pelletizing fungi, four were able to flocculate, but only three did so reliably (*G. lucidum*, *P. restrictum*, and *P. ostreatus*), removing 62–75% *E. gracilis* cells in a 1:2 (fungi:algae) suspension. In addition, the flocculation rate also exhibited species dependence with each species beginning to flocculate and arriving at T_{\max} at separate times. Flocculation is thought to be governed by the interactions of surface polysaccharides which are positively charged enabling static attraction to the negatively charged microalgae, and as the cell surface composition differs by species (Feofilova 2010), flocculation ability also differs (Ummalyima et al. 2017; Liber et al. 2020).

The differences in flocculation rates and times of arrival at T_{\max} could have been related to pH as it is known that pH affects flocculation via its effect on the Zeta potentials and hydrophobicity of both fungal and algal cells (Chu et al. 2021a). Under acidic conditions, the surface charge of algal cells becomes less negative, increasing destabilization of the cells in suspension (Bhattacharya et al. 2017; Wu et al. 2020). Fungi excrete organic acids which can help lower the pH, destabilizing the algae, and increasing the likelihood that the different cell types will be attracted to each other and flocculate (Chu et al. 2021a). Decreasing pH has also been found to affect hydrophobicity which in turn affects cell adhesion of fungal spores. Zhang and Zhang (2016) demonstrated that an increase in pH from 2.5 to 5.0 decreased the hydrophobicity of *Aspergillus niger* spores by 36%. It is probable that pH also has a similar effect on the hydrophobicity and adhesion properties of hyphae. Since hydrophobicity influences fungi-algae hydrophobic interactions (Chu et al. 2021a), lowering pH should increase hydrophobic interactions supporting the idea that lower pH favors flocculation and harvesting. Therefore, the ability of *P. ostreatus* to lower the pH of the combined fungi-algae culture should improve flocculation conditions resulting in faster and better flocculation. Based on the measured pH values, flocculation rates and arrival at T_{\max} should have had the following order: 1st *P. ostreatus*, 2nd *G. lucidum*, 3rd *P. restrictum*. However, though *P. restrictum*/*E. gracilis* flocs began to form last, its flocculation of the algae (as expressed by harvesting efficiency) was the highest. Meanwhile *P. ostreatus* was the

second to begin forming flocs with *E. gracilis* and had a lower harvesting efficiency. This conflict between observed and predicted results suggests that other factors are at play.

There was an inverse relationship between fungal dose and flocculation. At the highest concentration of fungi (1:0.5), there was no floc formation. Although the increased dose of fungi provided a larger surface area for algal attachment to hyphal surfaces, the large numbers of algal cells attached to and covering the surface of the filaments may have had a shielding effect. This could interfere with fungi-to-fungi self-adhesion and pelletization mechanisms, resulting in the loose floc formation observed. In general, the fungi-algae flocs formed by all three fungal species were loose and undefined forming aggregates rather than distinct pellets. Submerged mycelia do not have hydrophobins (Zhang and Zhang 2016), which may have led to reduced floc formation in our samples. Floc formation is a complicated process affected by many variables other than pH and species specificity, such as agitation, aeration, shear rate, media constituents, and ionic strength (Krull et al. 2013; Chu et al. 2021a). Therefore, it is difficult to pinpoint the exact reason for the observed loose floc morphology.

Harvesting efficiency

The harvesting efficiencies in this study were below the maximum ($\geq 90\%$) reported in the studies of Zhou et al. (2012); Wrede et al. (2014) and Chen et al. (2018), which employed the most widely studied methods of pellet- and spore-assisted microalgal harvesting. Those studies showed that harvesting efficiency depends not only on the fungal species but also on the size of the microalgae harvested. Wrede et al. (2014) compared the flocculation of eleven microalgal species of assorted sizes, motile and non-motile, by *Aspergillus fumigatus* pellets. The smallest (8–25 μm), non-motile, microalgae (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Scenedesmus quadricauda*) had the highest flocculation rates (up to $\sim 90\%$ in 24 h) while the largest (150–300 μm), non-motile, microalgae (*Pyrocystis lunula*) had the lowest flocculation rate (up to $\sim 40\%$ in 24 h). The results from our study suggest that large motile microalgae may not be harvested by fungal bioflocculation, either as filaments or pellets, as efficiently as smaller non-motile species (Ummalyima et al. 2017; Nazari et al. 2020). In a method similar to ours, Talukder et al. (2014) applied exogenous fungal filaments to immobilize *C. vulgaris* and *Nannochloropsis* spp., which are small ($\leq 10 \mu\text{m}$) non-motile algae. Their harvesting efficiencies (94–97%) for both algal species were notably higher than ours, further underscoring the difficulty of harvesting large motile species such as *E. gracilis*. However, Wrede et al. (2014) found that small (10–20 μm), but motile microalgae (*Tetraselmis chuii*, *Chlamydomonas reinhardtii*, and *Dunaliella salina*) flocculated at a lower rate

(~65–70% in 24 h) than similarly small non-motile species. In a study by Luo et al. (2019) using *P. ostreatus* pellets to rapidly (2.5 h) harvest *Chlorella* spp., the harvesting efficiency (64.9%) was similar to ours. Thus, it seems that in some cases motility and fungal species might be a greater determining factor than the size of the microalgae.

The differences in harvesting efficiencies between species and between different fungi to algae ratios of the same species show that harvesting efficiency is dependent on both species and fungi to algae ratio. The different harvesting efficiencies between the three species suggest species-specific attraction to *E. gracilis*. It is known that the species of the fungi and algae used affect harvesting due to species-specific cell wall properties in both organisms (Egede et al. 2016; Chu et al. 2021a). The fact that flocculation began at different times for each fungal species at the same ratio, but flocculation of the same species (*P. ostreatus*) at two different ratios occurred at the same time, points to species-specific differences in the strength and number of bonds formed. Li et al. (2006) found that the strength hierarchy of different flocculation bonds was bridging > charge neutralization > sweep. The strength of flocs formed and, therefore, harvesting efficiency will depend on which of the three mechanisms takes place. If the bioflocculation mechanisms between *G. lucidum* and *E. gracilis* formed weaker or fewer bonds than those of the other fungal species, then its ability to bind with and harvest the algae will be lower explaining the loose flocs and lower harvesting efficiency. Additionally, the static interactions between the differently charged surfaces of fungi and microalgae and active sites of polysaccharides on the filament surface allowing for bioadsorption affect the strength of the attachment of microalga to the fungal filaments (Zhang and Hu 2012; Wrede et al. 2014).

When *P. ostreatus* was added to *E. gracilis* at four different ratios, fungal dose and harvesting efficiency were positively correlated aligning with earlier findings (Li et al. 2020; Nazari et al. 2020). Harvesting efficiency at the lowest fungal ratio was 10–13% lower than for the other ratios which had similar removal rates, suggesting a minimum fungal inoculum concentration threshold for optimal microalgal harvesting. The inoculum spore concentration in spore-assisted and pellet-assisted microalgal harvesting affects floc formation and therefore harvesting efficiency, with lower spore concentrations inducing flocculation and harvesting in both harvesting types (Krull et al. 2013; Gultom et al. 2014; Zhao et al. 2019). However, in our case where hyphae were used, increased concentration led to suppressed floc formation but better harvesting efficiency. Talukder et al. (2014) obtained similar results in harvesting efficiency when fungal hyphae of *Aspergillus nominus* was used to harvest *Nannochloropsis* sp. In their study of the four ratios used, (4:1, 2:1, 1:1, and 1:2), the harvesting efficiency was above 80% for the highest three concentrations and noticeably lower at the

lowest fungal concentration (approx. 60%). Also harvesting time was longer at the lower mycelium concentrations (≥ 6 h vs. 3 h at 4:1 ratio).

Many other factors—agitation, pH, temperature, ionic strength, and constituents of the media—also affect harvesting efficiency (Krull et al. 2013; Chu et al. 2021a). Agitation speed is known to affect pellet formation and morphology which in turn affects microalgal harvesting efficiency. In a study by Luo et al. (2019), small, dense, smooth fungal pellets (formed at 150 rpm) were only able to capture microalgae via surface adsorption leading to poor harvesting efficiency. Meanwhile, mid-sized, loose, rough pellets (formed at 100 rpm) resulted in both internal and surface adsorption and therefore better harvesting. Similar effects of agitation on pellet morphology and its consequent harvesting efficiency have been well documented in other harvesting studies employing both pellet-assisted and spore-assisted methods (Bhattacharya et al. 2017; Zhao et al. 2019; Pei et al. 2021). It is possible that agitation may affect pellet morphology and microalgal flocculation and harvesting similarly when filaments in suspension are used. However, a more detailed study on the effects of various agitation speeds on the harvesting of *E. gracilis* using fungal filaments would need to be done.

As with pellet formation and flocculation (see discussion above), pH also impacts harvesting and lower pH tends to favor flocculation and harvesting (Zhou et al. 2012; Chu et al. 2021a). The starting pH of our harvesting experiments was neutral except for the combination of *E. gracilis* with *P. ostreatus* which was acidic. However, it was the combination of *P. restrictum* with *E. gracilis* which had the highest harvesting efficiency though it had the highest pH. This proves that microalgal harvesting is not always correlated with pH and that lowering pH does not necessarily result in improved harvesting. Similar results have been observed by Chu et al. (2021b). Flocculation and harvesting mechanisms are complex and varied and depend very much on the species (Egede et al. 2016; Chu et al. 2021a). In this case, species type likely exerted greater influence on harvesting than pH possibly due to the type of harvesting mechanisms at play. Another possible reason for this anomaly may be that although low pH encourages charge neutralization thus enhancing adhesion between fungal and algal cells, it may have a negative effect on other harvesting mechanisms lowering the overall harvesting efficiency (Chu et al. 2021b). *E. gracilis* is an acidophilic species suggesting that its zeta potential and resulting surface charge are likely still highly negative at low pH. This may explain the negligible effect of low pH on the harvesting efficiency of the microalgae in this study. Though pH is an important parameter of microalgal harvesting efficiency, we deliberately chose not to control or optimize pH as we wanted to produce a simple method which did not require parameter controls. It is also important

to note that pH is influenced by the medium's constituents. For instance, it has been found that increasing glucose concentration can lower pH (Zhou et al. 2012; Gultom et al. 2014). Therefore, if our microalgal harvesting method were to be employed using other media, the flocculation and harvesting efficiencies might differ from this study.

It is worth mentioning that temperature has a marked effect on harvesting efficiency. In general, temperature is directly correlated to harvesting efficiency, but only up to a maximal temperature specific to the fungal strain in question, after which it seems that heat stress damages the cells impeding harvesting (Bhattacharya et al. 2017; Khothari et al. 2017; Pei et al. 2021). Despite the possible harvesting efficiency gains of increasing temperature, this would necessitate energy inputs resulting in increased operational costs.

Harvesting time

In our study, the harvesting times were markedly shorter than most of the earlier studies (e.g., (Zhou et al. 2012; Al-Hothaly et al. 2015; Muradov et al. 2015), and T_{\max} was reached within 1–3 h for all three species (*G. lucidum*, *P. restrictum*, and *P. ostreatus*). Similarly, short harvesting times (2.5–3 h) have been reported for pre-pelleted fungi (Bhattacharya et al. 2017; Chen, et al. 2018; Luo et al. 2019) but only once for filaments (Talukder et al. 2014). In a study using fungal filaments to harvest and enhance oil production of *Nannochloropsis oceanica*, harvesting efficiencies were similar to ours (~60%) but at a much longer co-culture and harvesting time (6 days) (Du et al. 2018). In comparison to using pellet- and spore-assisted harvesting, this method of applying fungal filaments to microalgae in suspension is less laborious, bypassing many time-consuming preparation steps used in other methods like spore separation, counting, and pre-pelletization of the fungi (Talukder et al. 2014; Mackay et al. 2015; Luo et al. 2019). For instance, in studies with comparable harvesting results such as Wrede et al. (2014) and Luo et al. (2019), the preparatory step of fungal self-pelletization took 2 and 5–7 days respectively. In addition, Wrede et al. (2014) co-cultured the fungal pellets with microalgae for up to 48 h to achieve maximal harvesting. Whereas our method is faster and consumes less energy.

The interaction between flocculation and harvesting

Faster flocculation did not correlate with harvesting efficiency. For instance, although *G. lucidum* formed flocs and arrived at T_{\max} the fastest, it had the lowest harvesting efficiency of the three species. Meanwhile, *P. restrictum* had the third fastest flocculation rate and second fastest T_{\max} but had the highest harvesting efficiency of the three species. As discussed above, the extent of particle binding is related to the type and number of flocculation mechanism involved which

is species-dependent. If self-pelletization is caused by static attraction and/or hydrophobins (Linder 2009; Zhang and Hu 2012) which are, different mechanisms from microalgal flocculation caused by charge neutralization, patching, bridging, and sweep (Alam et al. 2016), then it makes sense that flocculation and harvesting rates do not necessarily correlate.

The short life span of *T. versicolor* flocs indicates that *T. versicolor* may quickly destabilize and flocculate microalgae via weaker bonding types, such as charge differences and entrapment, but produce insufficient EPS which are fundamental to the formation and structural maintenance of flocs (Nazari et al. 2020). The slight decrease in harvesting efficiency for all species after T_{\max} indicates that some *E. gracilis* cells “escaped” the fungi back into suspension, which could be due to charge difference, which is not enough alone to keep motile microalgae attached to fungal filaments as suggested by Egede et al. (2016). Other even stronger bonding types involving physical attachment of microalgae (*Nannochloropsis oceanica*) to fungal mycelia (*Mortierella elongata*) have been observed (Du et al. 2018). Such physical attachments would result in even stronger bonds and flocs than those caused by charge difference and EPS preventing escape of the microalgae.

Settling

Floc settling exhibited dosage dependency with floc settling improving with decreasing fungi to algae ratio. Although the highest ratio of *P. ostreatus* to *E. gracilis* (1:0.5) resulted in the highest harvesting percentage, the aggregates formed were loose, easily fell apart as separate filaments, and did not settle well or at all. The lowest dose of fungi (1:3) exhibited the lowest harvesting results but resulted in observably more dense aggregates which settled quickest and most compactly. This highlights the importance of considering settling ability when determining the optimal fungi to algae harvesting ratio. When the experiment was repeated at only two ratios, 1:1 and 1:2, settling results showed an obvious difference in settling rate and compactness, especially at 5 min. Although the difference between these two ratios was less than in the earlier experiment at four ratios, the difference (0.58 cm min^{-1}) was larger than the difference between different species (0.24 cm min^{-1}) at the same ratio. This shows that the fungi to algae ratio have a bigger impact on the settling rate than the species composition.

P. restrictum produced fungi-algae flocs which settled better than those produced by *G. lucidum* and *P. ostreatus*, indicating a species-specific effect on floc settling. Based on density observations, however, the densest, most discrete flocs (*P. ostreatus*/*E. gracilis*) would be expected to settle best, but it was the median dense flocs (*P. restrictum*/*E. gracilis*) that had the fastest settling rate. Microbial aggregates are highly permeable and porous allowing liquid to flow

through the aggregates reducing drag (Li and Yuan 2002). It is possible that because *P. restrictum*/*E. gracilis* flocs were less densely packed, they were more porous and therefore experienced less drag, settling better. However, in addition to species, density and porosity, size and shape (not measured here) also affect the settling ability of flocs (Jonasz and Fournier 2007; Benjamin and Lawler 2013). Furthermore, settling velocities for natural aggregates are also affected by particle concentration, mode of formation, and shear forces present (Hawley 1982; Gladman et al. 2005). Shear forces have a particularly large effect on the settling ability of natural flocs and are vastly different between lab-scale batch settling reactors and full-scale reactors. The latter usually contains more shear forces leading to more hindered settling and break-up of flocs (Gladman et al. 2005; van Deventer et al. 2011). Therefore, minor differences in settling ability between the flocs formed may be even more significant in a full-scale reactor than would be expected based on the results from the batch settling tests.

Practical implications

The method of harvesting applied in this study, provided the optimal fungi to algae ratio is used, could reduce the cost of microalgal harvesting by easing and lowering the energy demand of downstream dewatering steps. This idea is supported by the study of Fasaeei et al. (2018) which found that flocculation followed by a dewatering step consumed the least energy compared to other harvesting and dewatering methods. Furthermore, the low capital investments associated with flocculation could be further reduced using fungal flocculants as they lend themselves to growth in waste streams and allow for the recycling of growth media (Mishra et al. 2004; Chen et al. 2018). Instead of becoming a contaminant and hinderance to downstream applications, fungal bioflocculants add to the biomass' value by improving its total potential as a source of biofuels, biochemicals, and enzymes (Li et al. 2020; Chu et al. 2021a). Although a couple studies have applied fungal filaments similarly, they either have a lengthy harvesting time (Du et al. 2018) or are laborious and require specialized equipment (Talukder et al. 2014). Our method of direct application of fungal filaments in suspension removes the preparation steps required by other harvesting methods which further reduce both costs and time expenditure.

All these possible cost reductions make this an especially promising harvesting method for low-value microalgal biomass applications such as nutrient removal and recycling, feedstock for biofuel, feed, or fertilizer. The fungal harvesting method applied in this study did not harm *E. gracilis* which suggests that this method is compatible with microalgal biomass applications where

the health of the algae is important. In using previously untested combinations of fungal and algal species, this study adds to the current body of knowledge on fungal bioflocculation of microalgae. Further improvements in the harvesting efficiency of this method are possible but would need to be optimized as dictated by the specific system (type of growth media, reactor type, and local conditions (temperature etc.)) employed. Since the species of fungi and algae used also impact flocculation efficiency (Li et al. 2020), this system could also be improved by using other microalgal species, especially smaller non-motile species.

Conclusion

In this study, we demonstrated that harvesting of *E. gracilis* using fungal filaments can be a rapid and sustainable method. The harvesting efficiency was dependent on both the species and fungi to algae ratio while floc settling was mostly dependent on the latter. Out of the eight fungal species, the filaments of *G. lucidum*, *P. ostreatus*, and *P. restrictum* reliably flocculated and harvested *E. gracilis* with optimal harvesting and settling occurring at a fungi to algae ratio of 1:2. Harvesting of large motile microalgal cells with fungal filaments is relatively efficient, is rapid, and produces readily settling flocs, which can ease harvesting. In addition to rapid harvesting, it removes many of the laborious preparatory steps present in other methods. Altogether, these features can help to reduce harvesting costs while improving the total biomass' value. This is an important step forward in the effort to remove the economic and sustainability hurdles facing the commercialization of microalgal biomass for low-value applications.

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Data availability Available upon request.

Declarations

Conflict of interest The authors declare no competing interests.

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