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Short communication

A low cost, non-toxic biological method for harvesting algal biomass

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ABSTRACT

Microalgae are a popular source of feedstock for biofuels and used extensively in bioindustries. Although large-scale microalgal culture is relatively inexpensive there are bottlenecks that limit their usage in downstream applications. One of these is the harvesting process. Existing methods are expensive, make use of ecologically harmful chemical compounds, or limited to specific microalgae. This report describes and recommends a low-cost, non-toxic, biological method for harvesting algal biomass. In the presence of natural predators some microalgae aggregate. Here it is shown that the unicellular chlorophyte *Chlamydomonas reinhardtii*, a promising target organism for use in several bioindustries, flocculates when exposed to axenic cultures of the flagellate predator *Peranema trichophorum*. The predator's culture filtrate is even more potent: a dilution of 1:10 is sufficient to flocculate cells in half the time taken by *Peranema* cell suspension. Aggregates quickly settle to the bottom of bioreactors and can be harvested by dewatering or coarse filters. The method is potentially transferable to other commercially important chlorophytes like *Dunaliella* and *Chlorella*.

1. Introduction

Biodiesel is potentially an excellent alternative to fossil fuels; it is a renewable energy source and its combustion is typically environment friendly because of limited greenhouse gas emissions [1–3]. The agricultural biomass (ex., soybean, corn, sugarcane, palm oils, etc.) currently used as a feedstock for 1st generation biofuel production has several limitations [4,5]. Production requires significant agricultural resources (land, water) leading to increased costs. In addition, the crops are traditionally used as a food and feed source and their diversion for biodiesel production is controversial [6]. Microalgal biomass is an attractive alternative to offset some of these limitations [7–9]. Microalgal culture methods are relatively simple and cells can be grown indoors in large bioreactors or in arid land using open ponds containing fresh water, marine water or industrial effluents depending on the organism. The resultant biomass can be used directly or the culture conditions manipulated to accumulate excessive lipids for chemical conversion into biodiesel [1,2,10]. There are several chlorophyte species that may be employed in biorefineries or biofuel industries with the genera *Dunaliella*, *Chlorella* and *Chlamydomonas* currently the most promising candidate organisms [11–13].

Although microalgae are an attractive feedstock a major obstacle to increasing their usage is the unavailability of an economical, easy-to-use, scalable and eco-friendly method for harvesting the biomass generated [14]. Microalgae tend to remain suspended in culture because of their small size, motility, negatively charged surfaces and low cell densities. Current methodologies (ex., centrifugation, chemical flocculation, sedimentation, filtration, etc.) are either expensive, not useful for all types of algae or make use of chemicals whose removal further increases the cost, and if discharged untreated pollute the environment [14,15]. More recently biological methods (ex., co-culture of flocculating organisms with non-flocculating algae) have gained importance as these methods are effective and do not use toxic compounds. However, the cost of growing a flocculating organism for co-culture is a major drawback [9,16–18].

Despite the attention that the problem of harvesting has received costs remain high and range from 20–50% of the total cost from the step of biomass production to biodiesel manufacture [14,15,19]. Here a highly efficient, inexpensive and eco-friendly method for harvesting microalgal biomass is described. The culture filtrate of *Peranema trichophorum*, a natural predator of chlorophytes, was found to be a potent flocculating agent. The usefulness of this method was tested on the unicellular chlorophyte *Chlamydomonas reinhardtii*, which shows promise in biodiesel production, bioremediation, commercial gas production and more recently as a protein expression system [12, 20–23]. *Peranema* cells or their culture filtrate rapidly flocculated

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C. reinhardtii CC-125 cells with an efficiency of aggregation of >95%. The method is inexpensive, non-toxic and potentially applicable to other genera like *Dunaliella* and *Chlorella*.

2. Materials and methods

2.1. Organisms and culture conditions

C. reinhardtii CC-125 (*Chlamydomonas* resource centre, University of Minnesota, USA) is a commonly used model organism for biofuel production and strain development in biofuel related research. *P. trichophorum*, a natural predator of *Chlamydomonas* (our observations), was supplied by Carolina Biological Supply Company, Burlington, NC. *C. reinhardtii* CC-125 was grown in standard Tris–Acetate–Phosphate medium [24,25] at 22 °C in a 12 h:12 h light–dark cycle. *P. trichophorum* was grown at room temperature (22 °C ± 3 °C) with a natural day/night cycle. A simple inexpensive medium for *Peranema* culture was prepared by adding 2–3 wheat grains and 100–200 mg of boiled egg yolk to 100 ml spring water. The entire solution was boiled for 4–5 min and allowed to cool before inoculation with 0.5–1 × 10^4 *Peranema* cells. Sterile medium without exposure to predators or their culture filtrate is referred as *Peranema*-naïve growth medium. Within a week a large number of motile *Peranema* were observed and survived under these conditions for 2–3 weeks. A fresh *Peranema* culture was started by inoculating 1 ml old *Peranema* culture into 100 ml freshly prepared medium. Both *Peranema* and *Chlamydomonas* cultures were maintained axenically and checked every few days for contamination microscopically and by plating out 0.2 ml of each culture on Luria broth agar and incubation for 2–3 days at 22 °C and 37 °C.

2.2. Flocculation of Chlamydomonas cells

C. reinhardtii CC-125 was grown in 50 ml TAP medium in 250 ml Erlenmeyer flasks until late exponential to early stationary phase, adjusted to a cell density of 2 × 10^6/ml. A total 2 × 10^5 cells of *P. trichophorum* suspension was added to the flasks of *Chlamydomonas* cultures. In controls, 0.5 ml sterile *Peranema*-naïve growth medium was added. Flasks were incubated at 22 °C with constant shaking (140 RPM) in a growth chamber in a 12 h light: 12 h dark cycle. Flasks were monitored with the naked eye and microscopically every 12 h for flocculation. For microscopic observations a 10 μl sample was collected, loaded on a haemocytometer and observed using the 10× objective of a light microscope (Carl Zeiss, Axiostar, Ser Nr.34038). In another experiment, *Peranema* culture filtrate was tested as a flocculating agent to see whether the physical presence of predators was necessary for flocculation. *C. reinhardtii* CC-125 cells were grown using the culture conditions described above and inoculated in a six well plate (Nunclon surface; 35 mm diameter; ~15 ml) at a density of 1 × 10^7 cells/well. 2–3 weeks old *P. trichophorum* culture was collected and filtered using 0.2 μm pore size filters (Pall life sciences, South Africa) to remove living *Peranema* cells. The culture filtrate (0.5 ml/well to 4 ml/well) was added and the final volume adjusted to 5 ml using sterile TAP medium. The final *Chlamydomonas* cell density was 2 × 10^6 cells ml^{-1}. In controls either live *Peranema* cells (positive control) or sterile *Peranema*-naïve medium (negative control) was used. Plates were incubated at 22 °C in a 12 h:12 h light: dark cycle. Plates were observed after every 12 h with the naked eye or using the 10× objective lens of a light microscope (Olympus BX4-1 microscope and Carl Zeiss, Axiostar). The plates were photographed using a Sony digital colour camera (DSC-H90). A small sample was collected from the flasks/plates and unflocculated (unicellular) *Chlamydomonas* cells were counted using a haemocytometer. Loss of cells by predation prior to clumping was determined by average number of algal cells/predator (n = 25 predators) times total number of predators as a percent of total number of algal cells.

2.3. Naturally-occurring algal predators as flocculating agents

Water samples were collected from Emmarentia dam in Johannesburg, South Africa (26° 9′ 1″ S, 28° 0′ 21″ E) and potential algal predators were isolated with a micropipette. The aim was to determine whether naturally occurring freshwater samples containing predators (*Peranema* and other zooplankton) could also act as flocculating agents. Well-mixed water samples were collected in 50 ml Falcon tubes and immediately taken to the laboratory. 5 ml of the water sample was inoculated in a "zooplankton enrichment medium", which was prepared by boiling a few grains of wheat and rice in 500 ml spring water and cooled overnight. The flasks were incubated at room temperature (~23 °C) and the growth of zooplankton monitored daily using the 10× objective lens of a light microscope (Olympus BX4-1). The zooplankton were identified morphologically. To obtain pure cultures of one type of predator a small amount of mixed culture was placed in 15 cm culture plates and diluted using sterile water. Potential predators were located microscopically and transferred to fresh medium using a long glass micropipette. In some cases 2–3 rounds of dilution and transfer were required to obtain pure cultures. Several types of zooplankton were observed (ex., *Peranema*, *Coleps* sp., *Brachionus*, *Philodina*, and unidentified ciliates). *Brachionus* and an unidentified ciliate were subsequently tested as potential flocculating agents. 1 ml of each type of predator (~1 × 10^5 cells) was added to *Chlamydomonas* cell cultures as described above and flasks were incubated on a rotary shaker (22 °C, 140 RPM) in a 12 h light:12 h dark cycle.

3. Results and discussion

3.1. Natural predators induce flocculation in Chlamydomonas

The commercially purchased *P. trichophorum* was a natural predator of *Chlamydomonas*. *P. trichophorum* began engulfing single *Chlamydomonas* cells within 2 h of their co-culture. By 18 h, presumably as a defence against predation [26; our observation], almost all the *Chlamydomonas* cells (95.6 ± 0.6%; Mean ± SD; n = 4) had flocculated and sank to the bottom of the flasks/plates (Fig. 1B and H). The evolutionary ecology of this flocculating behaviour has been investigated further and will be reported elsewhere (manuscript in review). Flocculation was not observed in the control (Fig. 1A) where *Peranema*-naïve growth medium was used. This ruled out the possibility that the growth medium itself induced flocculation. The aggregated biomass settled rapidly and could easily be harvested by dewatering or using large pore size filters. The loss due to engulfed *Chlamydomonas* cells was insignificant (<3%) over the first few hours of monitoring and the presence of *Peranema* is unlikely to be an issue if the downstream application is biodiesel or biofertiliser production. Furthermore, *C. reinhardtii* CC-125 cells stayed in aggregates as long as the living predators were present (typically 8–10 days) giving plenty of time for biomass harvesting.

The *Chlamydomonas* cells also flocculated in the presence of a rotifer *Brachionus* and an unidentified ciliate (not shown). However, in this instance, both of these organisms were isolated from the natural water body described above and whether the stimulus for flocculation was predation or the presence of cryptic bacteria is unknown.

3.2. Peranema culture filtrate induces strong flocculation in Chlamydomonas

C. reinhardtii CC-125 cells also flocculated and formed large aggregates when *Peranema* culture filtrate alone (*Peranema* removed) was added (Fig. 1D–G). Flocculation in the presence of culture filtrate was even more rapid than the actual presence of *Peranema*; *C. reinhardtii* cells aggregated in 10–12 h whereas they took 18 h to aggregate when live *Peranema* were used. The effect was quantitative: more *Chlamydomonas* cells entered aggregates as the proportion of *Peranema* culture filtrate increased (Fig. 1D–G). The lowest volume tested (0.5 ml) effectively flocculated 1 × 10^7 *Chlamydomonas* cells. Filtrate volume less