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A rapid method for fungal assisted algal flocculation: Critical parameters & mechanism insights

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ABSTRACT

A method for rapid flocculation of *Chlorella pyrenoidosa* cells with *Aspergillus fumigatus* pellets was developed. The process could flocculate 99% algal cells within 3 h. In order to identify the critical parameters, apart from the flocculation conditions (different fungal-algal ratios, flocculation temperature and agitation), the effect of cultivation time and various pretreatments (autoclaving, Cycloheximide exposure) for *A. fumigatus* was also investigated. Results revealed that 24 h old fungal pellets flocculated at 38 °C and 1:5 fungal-algal ratio showed the best flocculation efficiency. The cell viability assay showed that a viable and metabolically active fungal pellet is a prerequisite for flocculation. Scanning Electron Microscopy (SEM) studies confirmed that in addition to viability, an intact and undamaged hyphae is also required for algal attachment. Fourier transform infrared spectroscopy (FTIR) data of the algal-fungal pellets compared to that of algae and fungi showed the involvement of specific groups in the interaction. Sharp decrease in peak intensity at 1024 cm^{-1} for the algal-fungal pellets indicated the role of C-N groups in the flocculation process. The lipid content of the harvested algal fungal pellet was similar to the algal and fungal partners. Finally, this method was tested on wastewater grown algae, where 95% flocculation was achieved within 3.5 h. The algal-fungal pellets (1650 μm diameter) could be easily separated from the treated water. Hence, this process could serve as an alternative for concentrating microalgal cultures for biofuel production in a cost effective way. This report reveals critical parameters and new insights on algal-fungal flocculation apart from providing a rapid and feasible algal harvesting technique.

1. Introduction

In spite of immense potential of microalgae for wastewater treatment as well as bioenergy generation [1–4], costly and energy intensive harvesting limits practical application of this eco-friendly approach towards solving the wastewater and energy issues [5,6]. The choice of appropriate harvesting method is a challenging task, especially during simultaneous wastewater treatment and biofuel production, where both the acceptable quality of treated water as well as suitable composition of the harvested algal biomass is to be ensured. Although physical processes are rapid, they are energy intensive which significantly increases the cost of wastewater treatment and biofuel production [5]. Chemical methods, though less expensive than the physical ones, contaminate the microalgal biomass as well as treated water with undesirable chemicals [7]. Use of flocculants and alkali for harvesting has to be reversed or neutralized before taking the biomass for fuel production [8,9]. In this context, bioflocculation process is of great interest since it is a chemical free method and flocculation occurs spontaneously after the addition of a biological agent without the input of external energy unlike physical flocculation processes. Moreover, the biologically harvested biomass is more suitable for biofuel production since bioflocculation has been reported to increases the lipid content of the harvested biomass [10].

Bioflocculation of algae has been previously performed by employing suitable microbial partner viz. algae-algae, algal-bacterial and algal-fungal interactions [11–13]. Co-cultivation of pellet forming filamentous fungi with microalgal biomass has been recently reported as efficient algal harvesting technique [12,14–17]. Fungal harvested microalgal biomass can be successfully used for biofuel production. Al-Hothaly et al. [18], harvested *Botryococcus braunii* biomass from 500 L culture using *Aspergillus fumigatus* and the resultant biomass was used for biodiesel production by hydro pyrolysis. A wide range of morphologically and physiologically different microalgal cells were harvested using co-cultivation with *A. fumigatus* by Wrede et al. [17]. However, major problems associated with co-cultivation mode are inhibition of fungal growth due to high pH of algal culture, requirement of carbon source and longer

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cultivation time of 24–48 h [12]. Moreover, the mechanism of algal-fungal flocculation and the critical parameters governing the same are poorly understood. These aspects need more attention in order to develop the fungal assisted algal flocculation in the most efficient manner.

Recently, our group reported rapid harvesting of *Chroococcus* sp.1 by pre-cultivated pellets of *Aspergillus lentulus* within 6 h. The process resulted in simultaneous bioflocculation and enzymatic pretreatment of algae, thereby significantly improving the digestibility and methane yield from harvested biomass [19]. Use of pre-cultivated fungal biomass overcomes the above mentioned problems associated with algal-fungal co-cultivation process. This approach opens up the possibility of using waste fungal biomass from fermentation industries as a flocculent and accords flexibility to manipulate the fungal-algal ratio as well as cultivation age of fungal pellets independently of the algal cultures. In the present study, pre-cultivated *A. fumigatus* pellets were used for the flocculation of *Chlorella pyrenoidosa*. In order to identify the critical parameters, apart from the flocculation conditions (fungal-algal ratios, flocculation temperature and agitation rate), the effect of cultivation time and various pretreatments (autoclaving, Cycloheximide exposure) of *A. fumigatus* pellets was also investigated. Finally, the process was validated for harvesting different microalgal cultures as well as wastewater grown *C. pyrenoidosa* biomass.

2. Materials and methods

2.1. Microorganisms and growth conditions

Microalgal species *C. pyrenoidosa* was obtained from National Collection of Industrial Microorganisms (NCIM), NCL Pune (India). *Chroococcus* sp1 (CC1) was isolated by Prajapati et al. [20] and a native consortia isolated from a nearby lake (Hauz Khas, New Delhi, India) was named as HL. Algal cultures were maintained in 2% algae cultivation agar (HiMedia M343-500G) slants supplemented with BG11 (HiMedia, M1541-500G) in a plant growth chamber (Daihan Labtech, LGC-5101). Liquid cultures were maintained in BG11 broth. For experimental purposes, microalgal culture was grown in 2.5 L flasks under continuous light in greenhouse maintained at 25 ± 1 °C and a light intensity of ≈2500 Lux.

A. fumigatus previously isolated in the lab was used. Fungal culture was maintained on sterile potato dextrose agar (PDA) slants (Himedia M096-500G) at 28 °C. Fungal spore suspension prepared with 0.1% Tween-80 ($\approx 10^8$ spores ml^{-1}) was inoculated in 100 ml potato dextrose broth (PDB; Himedia M403-500G) and incubated for 24 h at 28 °C and 150 rpm in an orbital shaker.

2.2. Flocculation experiments

Flocculation experiments were carried out in 250 ml conical flasks at 38 °C (unless otherwise stated) according to the patented process [21]. Pre-grown fungal biomass (7 g L^{-1} biomass concentration) cultivated at 28 °C for 24 h was added in 100 ml of algal culture (Optical Density [OD_{680}] 2–2.5 ≈ 0.98 g L^{-1} biomass concentration) at 1:5 fungal-algal ratio (on dry weight basis) and agitated at 100 rpm (unless otherwise stated) in an orbital shaker for 4 h. Samples were drawn every 30 min and OD was measured at 680 nm. The flocculation efficiency (FE) was calculated as [17]:

$$FE = \left(1 - \frac{OD_t}{OD_0}\right) * 100$$

where FE = flocculation efficiency at time t, OD_t = optical density at time t and OD_0 = initial optical density.

The FTIR spectra of algal and fungal biomass as well as algal-fungal pellet (collected after 4 h of flocculation) were analyzed to study the surface groups involved in algal-fungal interaction.

2.3. Algal and fungal surface characterization and biochemical composition

The algal cells were first diluted to an optical density of 0.15–0.20 at 680 nm (OD_{680}) with BG11 media. The pH of the solution was adjusted by adding either 0.1 mol L^{-1} NaOH or 0.1 mol L^{-1} HCl solutions [22]. Zeta potential measurements of algal cells were done in various pH at room temperature (Malvern Zetasizer Nano ZS90 + MPT-2).

For Fourier transform infrared spectroscopy (FTIR) measurements of algae, fungus and algal-fungal pellets, the samples were first washed with phosphate buffer saline (PBS) and then lyophilized (Allied Frost FD3). The lyophilized powder was then used for FTIR analysis using a Nicolet Is50 (Thermo Scientific) instrument.

A known amount of algal cells (0.10 g) were harvested by centrifugation and washed with PBS. The cells were then resuspended in 0.1 M $NaNO_3$ buffer. Potentiometric titration of the algal cells was done using Eutech CyberScan PC510 pH probe under continuous N_2 sparging as outlined by Hadjoudja et al. [23]. The acid-base titration data was modeled with Protofit ver 2.1 rev 1 [24] to determine the protonation constants.

The protein and lipid content of algal, fungal and algal-fungal pellets were measured. For lipid estimation, 0.50 g of dried biomass was taken and lipid was estimated by extracting with chloroform and methanol [20]. Protein estimation was done from 10 mg of lyophilized biomass. The samples were first resuspended in 200 μl of protein extraction buffer (1% SDS; 9 M Urea; 25 mM Tris-Cl pH 6.81; 1 mM EDTA and 0.7 M β-ME) and sonicated for 5 min with 30 s pulse. The samples were then centrifuged at 10,000 rpm for 10 min and the supernatant was used for protein estimation using Bradford's method [25].

2.4. Identification of the critical parameters and plausible mechanism of rapid flocculation

To identify critical parameters for rapid flocculation, two types of studies were conducted. In the first set of studies, the effect of pretreatments and age of fungal pellets prior to mixing with algae was evaluated while in the other, the effect of process parameters during flocculation was studied.

2.4.1. Effect of fungal cultivation time and pretreatments

The effect of cultivation time for fungal pellets was evaluated by cultivating *A. fumigatus* at 28 °C for 24 h and 72 h. Algal flocculation was carried out at 38 °C and 100 rpm as outlined in Section 2.2. Fungal pellets cultivated for 24 h and 72 h were subjected to scanning electron microscopy (SEM) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) analysis for studying the change in surface properties and viability of the fungal pellets, respectively. To further confirm the role of fungal viability in algal bioflocculation, fungal pellets cultivated at 28 °C for 24 h were subjected to autoclaving and Cycloheximide pretreatment. The fungal pellets were treated with Cycloheximide (10 μg ml^{-1}) for 2 h at 28 °C and 150 rpm. The treated pellets were then used for flocculation experiments as described in Section 2.2. Similarly, fresh fungal pellets (24 h old) were heat killed by autoclaving at 121 °C and 15 psi for 15 min and the resulting fungal biomass was used for the flocculating experiments. The FTIR of untreated, Cycloheximide pretreated and autoclaved fungal pellets was also conducted to study the effect of treatments on surface groups.

2.4.2. Effect of flocculation parameters (flocculation temperature, fungal-algal ratio and agitation)

The effect of flocculation temperature (28 °C, 30 °C, 32 °C, 34 °C, 36 °C, 38 °C and 42 °C) on FE was studied at a fungal: algal ratio of 1:5 using fungal pellets cultivated at 28 °C for 24 h. MTT assay of the algal-fungal pellets flocculated at 28 °C and 38 °C was performed to study the difference in metabolic activity at these temperatures. Further, to check the variation in metabolic activity of the fungi at different temperatures, MTT assay of the fungi was carried out at 28 °C and 38 °C.