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Flocculation of wall-deficient cells of *Chlamydomonas reinhardtii* mutant *cw15* by calcium and methanol

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ABSTRACT

Flocculation is a common and inexpensive method for harvesting algae from solution. After nitrogen starvation, it was shown that 83 ± 3% of the wall-deficient cells of the *cw* 15 mutant of *Chlamydomonas reinhardtii* flocculated from 12 mL samples within 15 min after the addition of 15 mM calcium chloride at pH 8.4. Only 24 ± 2% of the wildtype strain flocculated under these conditions, thus demonstrating how a simple mutation might facilitate process design. The data suggested that algae grown in waters with similar calcium concentrations (e.g. certain wastewaters) might be harvested through simple pH adjustment. It was also discovered that the addition of small amounts (<5% v/v) of methanol could significantly reduce the calcium needed to achieve flocculation. Within 15 min after addition of 12 mM calcium chloride and 4.6% (v/v) methanol, 83 ± 4% of *cw15* cells flocculated. Methanol is fully recoverable by distillation, and its use might enable flocculation without further water salinization when media calcium concentrations fall short of 15 mM. It was further shown that substrates for and/or products of cellular growth affected flocculation adversely. Nearly 81% of cells flocculated from fresh medium compared to only 54% in spent medium.

1. Introduction

Algae are widely considered among the premier candidate organisms for the production of biofuels. Researchers have coaxed numerous species into generating biodiesel [1–5], biohydrogen [6], biomass for burning [7], and even bioethanol [8] with results that compare very favorably to generating fuels from higher plants.

The algal species *Chlamydomonas reinhardtii* had previously been disfavored for biodiesel production on account of its minimal accumulation of the triacylglyceride (TAG) precursors required for diesel production [9], [10]. However, under nitrogen starvation conditions, TAG production was recently demonstrated to increase 15-fold in wildtype and 30-fold in the *cw15 sta6* mutant to levels competitive with other species. These TAGs comprised mostly unsaturated and mono-unsaturated fatty acids with chain lengths of 16–18 carbons, which should yield a good quality biodiesel [9].

Though other species of algae have proven to be more efficient producers of TAGs, *C. reinhardtii* mutants *cw15 and cw15 sta6* offer some distinct advantages. Firstly, *C. reinhardtii* is one of the best characterized of all algal species [11], [12]. Its physiology has been explored extensively, it has a fully sequenced genome, and it has proven amenable to genetic engineering. Secondly, the aforementioned mutants have no cell wall [9]. Algal cell walls provide not only a barrier against the environment, but also against engineered processes aimed at TAG extraction. The absence of this barrier would

Abbreviations: TAP, tris acetate/phosphate buffer; TAG, triacylglyceride.

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likely make TAG extraction substantially easier than in microalgae with intact cell walls. This has, in fact, been demonstrated in *cw15* and *cw15 sta6*, where lipid bodies are excreted upon drying [9].

Numerous techniques have been employed to harvest algae from solution, including flocculation [13–16], ultrasonic aggregation [17], centrifugation [18], froth flotation [19], filtration [20] and simple settling. Each has characteristics that become more salient in particular situations. Centrifugation, for example, is fast and highly effective, but also energy intensive and not economically viable for unicellular algae [21]. Settling is often slow and only works on larger diameter cells, but requires no energy input [22].

Flocculation refers to a conjoining of particles or cells in a suspension, usually initiated by a coagulant. It is a common water treatment technique for removing contaminants from solution and can be used as a stand-alone process or as a pretreatment prior to centrifugation. A number of coagulants have been used to target specific algaculture solutes, including multivalent metal salts, polyelectrolytes, and chitosan [13], [16], [19]. Flocculation can also be achieved in some species by altering environmental conditions, such as increasing pH [23].

Choosing the appropriate flocculating agent for an algae biodiesel process requires an appreciation for the overall process if the environmental and economic costs are to be minimized. For example, increasing pH (usually to 10 or higher) may induce algal flocculation, but the supernatant must be neutralized before it is recycled or released into the environment. Water salination is a major concern of water planners, so avoiding the use of neutralizing acids and coagulating salts is environmentally judicious [23]. Additionally, residual coagulant can complicate water recycling. Divikaran and Pillai (2002), for example, found that residual chitosan from the filtered supernatant of a flocculated algal culture continued to flocculate algae in cultures grown later in the recycled water [14].

Trial and error led us to discover that calcium chloride works effectively as a flocculant of *cw15*. Sukenik noted that at elevated pH (pH 9), calcium co-precipitates with the algae *Scenedesmus falcatus* [15]. The calcium-induced precipitation we observed took place at pH 8.2–8.4 in a medium that also contained phosphate. Serendipity further revealed to us that methanol could replace some of the calcium needed to flocculate *cw15*. Unlike other coagulants, methanol is fully and easily recoverable from aqueous media through distillation and is typically on-hand in biodiesel processing facilities (for the transesterification of TAGs to diesel). The objectives of the current study, then, were to characterize the flocculation of *cw15* and wildtype cells using various combinations of calcium chloride and methanol. We also sought to determine whether any inhibitors of flocculation might exist in the algae medium.

2. Materials and methods

2.1. Cell culture

Wildtype (C137) and *cw15* (CC-4349) *C. reinhardtii* were obtained via the Chlamy Center at Duke University. In all experiments, cells were grown with stirring in flat-sided, 1 L flasks of tris acetate/phosphate medium until they reached a chlorophyll concentration of 20–25 mg/L. Cells were then transferred to a set of 50 mL conical tubes and centrifuged at 1200 g for 5 min. They were then diluted and kept in nitrogen-free TAP media at a concentration of 16–18 mg/L for 48 h. Initial media pH was adjusted to 6.8 with HCl in both types of media prior to autoclaving. Cool white fluorescent lighting of 100 ± 10 $\mu mol/m^2/s^2$ was provided on a 12 h light/dark cycle. The TAP supernatant was collected and frozen upon transfer to nitrogen-free media.

2.2. Flocculation experiments

2.2.1. Calcium/methanol experiments

For each treatment, 65 mL of nitrogen-starved wildtype or *cw15* cells were transferred to a 100 mL beaker and stirred with a stir bar at 250 rpm on a stir plate. A 5 M stock solution of calcium chloride was prepared and methanol was added from a neat preparation. First, the effect of calcium concentration alone was evaluated using 0, 8, 12, 15, 31, and 62 mM calcium chloride. Then the effectiveness of flocculation of various calcium and methanol concentrations was assayed according to a full factorial design. Calcium concentrations were adjusted to 0, 8, 12, 15 mM and methanol concentrations were adjusted to 0, 1.2, 2.3, and 4.6% (v/v). After addition of a given combination of calcium and methanol to a given beaker of cells, the solution was mixed for 5 min prior to transfer. 12 mL aliquots were sampled from the beaker and transferred to 15 mL disposable test tubes via a pipetman set to slow speed and equipped with a 25 mL pipette tip. Flocs in these aliquots were allowed to settle for 10 min. After 10 min, 10 of the 12 mL sample was transferred to a new test tube. Each experimental treatment, then, comprised two aliquots containing 2 mL of settled cells and 10 mL of suspended cells. These were each measured for chlorophyll content. The fraction of cells flocculated was defined as the amount of chlorophyll in the bottom fraction divided by the total chlorophyll in both fractions.

2.2.2. Media composition experiments

Nitrogen-starved *cw15* cells grown as above were transferred to a set of 50 mL conical tubes and centrifuged at 1200 g for 5 min. Cells were then resuspended in an equal volume of one of four types of media: the same nitrogen-free media that they had been harvested from (hereafter called "nitrogen-free"), nitrogen-free media to which 0.4 g/L ammonium chloride was added (hereafter "nitrogen-free + N"), fresh TAP media, or TAP media in which cells had grown for 48 h. In each case, the medium was adjusted to pH 8.4 with potassium hydroxide (KOH) prior to resuspension. Flocculation experiments were then carried out as above in each media type using 12 mM $CaCl_2$ and 1.2% methanol as the flocculant.

2.2.3. Water recycling experiment

To test whether supernatant water from flocculation would require desalination before being recycled for growing cells, *cw15* cells were grown as above but were harvested during exponential growth, when their concentration reached about 18 mg/L chlorophyll. These cells were resuspended in four separate beakers to 17 mg/L in fresh TAP media (65 mL) with a pH of either 6.9 or 8.4 (two beakers each). The latter pH was