

2011

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Scholz, Matt; Hoshino, Takanori; Riley, Mark R.; and Cuello, Joel, "Flocculation of wall-deficient cells of *Chlamydomonas reinhardtii* mutant cw15 by calcium and methanol" (2011). *Biological Systems Engineering: Papers and Publications*. 439.
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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 $83 \pm 3\%$ of the wall-deficient cells of the *cw15* 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, $83 \pm 4\%$ 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.

Keywords: Biofuel Flocculation Methanol Algae *Chlamydomonas reinhardtii* *cw15*

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

Introduction

Algae are widely considered among the premier candidate organisms for the production of biofuels. Researchers have coaxed numerous species into generating biodiesel [1e5], 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 monounsaturated fatty acids with chain lengths of 16e18 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 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 [13e16], 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 8.2e8.4 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.

Materials and methods

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 nitrogenfree 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 $100 \pm 10 \text{ mmol/m}^2/\text{s}^2$ was provided on a 12 h light/dark cycle. The TAP supernatant was collected and frozen upon transfer to nitrogen-free media.

Flocculation experiments

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. Floccs 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.

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 p 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.

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 achieved through

addition of KOH. For the flocculation tests, one beaker from each pH regime was used as a negative control and 15 mM CaCl_2 was added to the other. Flocculation efficiency was measured as above.

Chlorophyll measurements

Test tubes of “unsettled” and “settled” cells were spun at 1100 g for 5 min to collect the cells. In each case, the supernatant was decanted and the residual cells were resuspended in an equivalent volume of neat ethanol. Cells were manually dispersed in the ethanol solution using a glass rod before being centrifuged at 1100 g for 5 min. The UV absorbance of the supernatant was obtained at 649 and 665 nm (Ocean Optics) and the chlorophyll in the fraction was calculated by the equation [11]:

Total chlorophyll (mg/L) $\frac{1}{4} 6.1 \cdot A_{665} \div 20.04 \cdot A_{649}$. Three measurements were taken on each of the four samples (8 fractions) of a given treatment. These measurements were averaged for each fraction. Flocculation efficiency was defined as the ratio of the chlorophyll measured in the bottom fraction and the total chlorophyll from both the bottom and top fractions of each sample. In this way, 4 replicate measurements of flocculation efficiency were obtained for each treatment tested.

Statistical analysis

ANCOVA and pairwise-t-tests were performed using the R platform for statistical computing [23]. Outliers were removed using a built-in outlier detection function.

Results and discussion

Effect of calcium chloride on flocculation

The specific *cw15* mutant of *C. reinhardtii* used lacks a cell wall and has no flagella for motility. We were interested in how this phenotype affected the tendency of the cell to flocculate in the presence of calcium. CaCl_2 was tested as a flocculant of nitrogen-starved wildtype and *cw15* cells (Fig. 1).

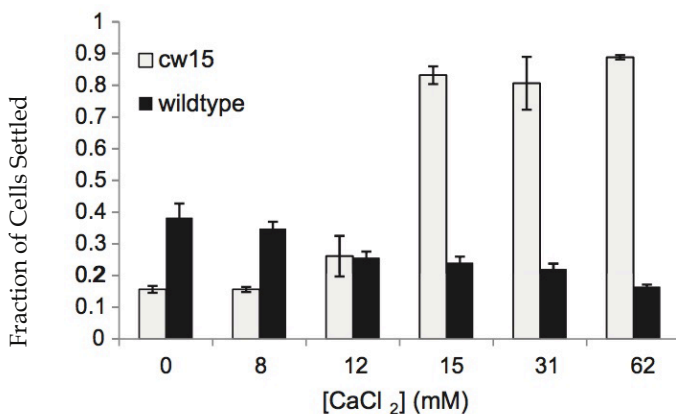


Fig. 1 - Flocculation with calcium chloride. Comparison of the effect of calcium chloride addition on flocculation of wildtype and *cw15* *C. reinhardtii* cells. Media was 48-hour- old, nitrogen-free TAP. Error bars reflect one standard deviation from the mean (n [4).

Wildtype cells were largely unresponsive to treatment and may have actually settled less as calcium concentrations increased. In contrast, the cw15 cells readily flocculated with the addition of CaCl_2 in excess of 12 mM. There appeared to be a critical concentration between 12 and 15 mM at which cells began to settle out of solution. The addition of more than 15 mM CaCl_2 did not improve flocculation under the assay's conditions.

X-ray imaging work performed in a previous study determined that, at pH 8.5, calcium and phosphate coordinated to form what appeared to be octacalcium phosphate, and that this was a precipitating agent for the algae *S. falcatus* [15]. The media used in the present experiments also contained phosphate ions, so a similar mechanism may have been at play. It is interesting to note that, like wildtype *C. reinhardtii*, *S. falcatus* has a cell wall, whereas cw15 does not. This might suggest that it is not the lack of cell wall per se that permits cw15 flocculation, but perhaps its consequent lack of flagella.

Table 1 - Analysis of Covariance.

The effects of the concentrations of calcium and methanol on flocculation of cw15 cells were characterized by an ANCOVA model that comprised two first-order terms and a second-order interaction term. a). Experimental design matrix. Four concentrations of each calcium chloride (0–15 mM) and methanol (0–4.6% v/v) were combined in a full factorial design. Each letter represents a specific combination of the two chemicals. b). Summary of ANCOVA results

Calcium Chloride Concentration (mM)					
Methanol Concentration (%v/v)	0	8	12	15	
	A	B	C	D	
	E	F	G	H	
	I	J	K	L	
	M	N	O	P	
Response: fraction of settled cells					
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
calcium	3	4.9778	1.65927	1478.3434	< 2.2e-16
methanol	3	0.0384	0.01281	11.4156	1.234e-05
calcium*methanol	6	0.0600	0.01000	8.9066	2.503e-06
Residuals	43	0.0483	0.00112		
Residual standard error: 0.0335 on 43 degrees of freedom; Multiple R ² : 0.9906, Adjusted R ² : 0.988; F-statistic: 376.9 on 12 and 43 DF;					

Effect of calcium chloride and methanol on flocculation

An accidental discovery suggested that methanol facilitated calcium-induced flocculation of cw15 cells. This inspired a full factorial experiment in which various concentrations of methanol and CaCl_2 were added to solutions of nitrogenstarved cells from the wildtype and cw15 lineage (Table 1 and Fig. 2).

Neither calcium nor methanol appeared to positively affect flocculation in wildtype cells and, in fact, may have reduced their tendency to aggregate. Under each condition, including the control, approximately 20e40% of the wildtype cells settled out of solution in 10 min.

Methanol alone also did not appear to affect flocculation in cw15 cells beyond what was observed in the no-methanol control (w15% in 10 min). This was the case even at much higher concentrations of methanol than those tested here (data not shown). However, not only did increasing concentrations of calcium greatly improve settling in cw15, but so did increasing methanol concentrations in combination with calcium. This effect was not large until the methanol concentration reached 4.6% (v/v). Higher concentrations of methanol were not assayed owing to concerns that they would remove chlorophyll from the cells and thus confound these tests.

The most observed settling was for a combination of 15 mM CaCl_2 and 4.6% methanol (Table 1, condition P), where $98 \pm 1\%$ of the cells had settled from solution in 10 min. This was more settling than the $89 \pm 1\%$ achieved with 62 mM CaCl_2 CaCl_2 and no methanol. Interestingly, the use of 12 mM CaCl_2 with 4.6% methanol (condition O) induced

as much flocculation as did 15 mM CaCl_2 alone (condition D). Also, more than 70% flocculation was achieved with 8 mM CaCl_2 and 4.6% methanol (condition N). Ethanol was observed to yield a similar effect, though the resulting flocs appeared more susceptible to disaggregation (data not shown).

By comparison, Oh et al. compared the effectiveness of three types of flocculant on *Chlorella vulgaris*: a *Paenibacillus* bioflocculant, aluminum sulfate (2 mg/L), and polyacrylamide (2 mg/L) [24]. They found flocculation efficiencies (defined and tested similarly to the present study) of 83%, 72%, and 78%, respectively, and determined that the bioflocculant worked best in the presence of 6.8 mM calcium chloride.

ANCOVA was performed to characterize effects of calcium and methanol on cw15 flocculation (Table 1). A regression model of the following form was fit with an adjusted R^2 of 0.99: where the explanatory factors were modeled as categorical variables. The response variable, "fraction settled," refers to the amount of chlorophyll measured in the bottom 2 mL of a given test tube divided by the total chlorophyll in the tube. It is described by two explanatory variables representing the calcium and methanol concentrations assayed. The "calcium*methanol" term describes a physical interaction between calcium and methanol that increases flocculation (see below). Each term was positive (i.e. each variable promoted flocculation) and was found to be significant to $p \ll 0.001$. The ε term represents error, where $\varepsilon \sim N(0, \sigma^2)$

The model suggests a second-order effect caused by the interaction of calcium and methanol. It has been previously shown that in an aqueous solution of 5 mol% methanol, the average number of hydrogen bonds formed per water molecule decreases significantly from that observed in pure water [25]. Perhaps a description for the mechanism by which methanol enhances flocculation can be sought here, though it should be noted that the concentrations of methanol in the present study never exceeded about 2 mol%.

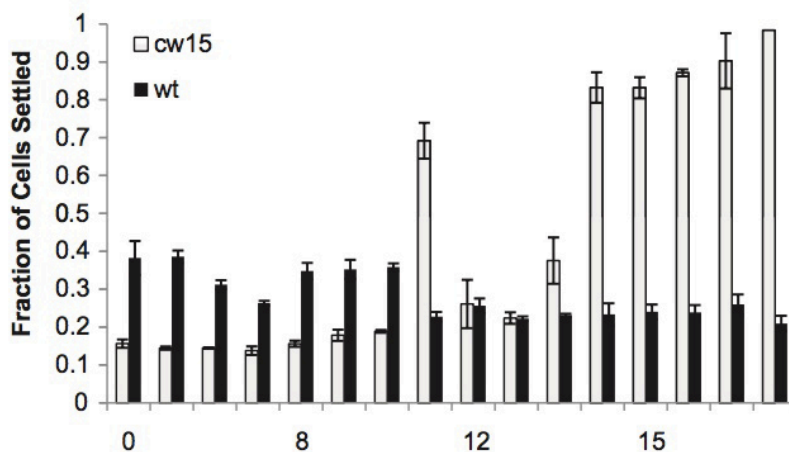


Fig. 2 - Comparison of the combined effects of calcium and methanol on the flocculation of wildtype and cw15 *C. reinhardtii* cells. The effect of an increasing series of calcium chloride on flocculation of both cell types was observed. Media was 48-hour-old, nitrogen-free TAP. Each of the four pairs of bars within a given category of CaCl_2 concentration (0, 8, 12, or 15 mM) represents a methanol concentration. These methanol concentrations increase from left to right within each CaCl_2 group: 0, 1.2, 2.3, and 4.6 % (m/v). For example, the sixth set of bars from the left represent CaCl_2 [8 mM, MeOH [1.2%. Error bars reflect one standard deviation from the mean ($n = 4$).

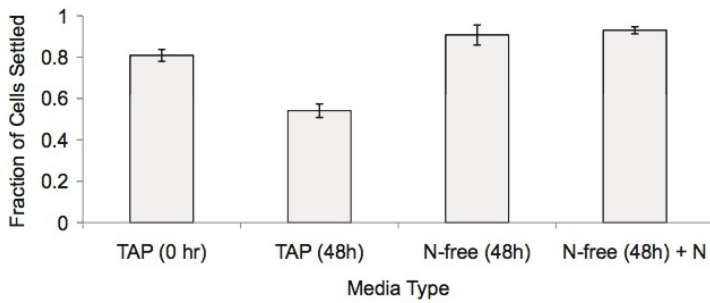


Fig. 3 - Effect of media composition on flocculation. Comparison of flocculation of cw15 in four types of media after addition of 15 mM CaCl_2 . Media types are: fresh TAP media (TAP), TAP media after 48 hours of growth (TAP (48 h)), nitrogen-free media after 48 hours of nitrogen starvation (N-free (48 h)), and N-free media to which a concentration of ammonium chloride equivalent to that found in fresh TAP media was added (N-free (48 h) D N). Error bars reflect one standard deviation from the mean ($n = 4$ replications).

The difference in response between wildtype cells and cells of this cw15 mutant is most easily explained by the absence of flagella in cw15 mutants. Non-motile cells would intuitively seem more amenable to aggregation than motile ones. However, we cannot rule out other possibilities. For example, the cell wall of wildtype cells may simply bear a weaker negative charge than the exposed cell membrane of cw15.

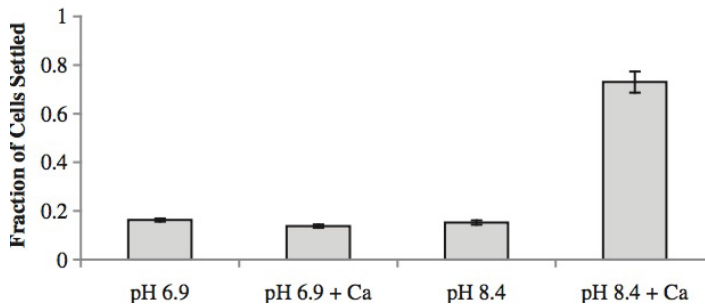


Fig. 4 - Requirement of elevated pH for flocculation. Growth-phase cw15 cells were resuspended to a concentration of 17 mg/L chlorophyll in 2 beakers each of either pH 6.9 or pH 8.4 media. One beaker of each set was left as a negative control, while 15 mM CaCl_2 was added to the other. After five minutes of stirring, cells from each beaker were transferred to a set of 4 test tubes where they were allowed to settle for 10 min. Chlorophyll measurements of both the top 10 and bottom 2 mL of these test tubes were taken to determine the fraction of settled cells. Error bars reflect one standard deviation from the mean ($n = 4$ replications).

Effect of media composition on flocculation

Nitrogen starvation can be achieved in batch culture either by replacing the nitrogen-containing media with nitrogen-free media (which requires cell harvest), by permitting cells to consume all of the nitrogen, or by sequestering the nitrogen so it is unavailable for consumption. To test whether the first two methods produce disparate flocculation results, nitrogen-starved cw15 cells were resuspended in one of four types of media and treated with 15 mM CaCl_2 (see Section 2.2.2).

The results (Fig. 3) demonstrate that in terms of flocculation there was no significant difference between media from the end of nitrogen starvation and media from the end of nitrogen starvation that had been supplemented with a concentration of ammonium chloride equal to that used in fresh growth media ($p = 0.36$). In other words, the presence of nitrogen did not affect flocculation significantly. There was a small but significant ($p < 0.01$) decrease in the fraction of cells flocculated in fresh TAP media as compared with either of the nitrogen-free media. However, the largest observed difference was between used growth media (i.e. TAP media after 48 h growth) and the other treatments, especially the nitrogen-free ones. Whereas more than 90% of the cells in the nitrogen-free media settled during the assay, only 54% did so in the used growth media.

The result that the nitrogen-free media were more conducive to flocculation than fresh TAP medium suggests that perhaps a solute inhibitory of flocculation in TAP medium was consumed by the algae and/or a metabolic byproduct of stationary phase growth in nitrogen-free media promotes flocculation. Additionally, the result that fresh TAP medium was better suited for flocculation than used growth medium suggests that a solute that was being consumed during growth promoted flocculation and/or a metabolic byproduct of growth inhibited it.

Combined, these results lead us to conclude that to affect nitrogen starvation, it is preferable to replace the growth medium with a nitrogen free medium rather than simply allow the cells to exhaust their nitrogen supply or remove the nitrogen from solution. In practice, media replacement requires some method of cell harvesting. An alternative to media replacement would be to either remove or replenish the compound(s) in the used growth media that inhibit or enhance flocculation, respectively. Mucopolysaccharides in cyanobacteria are known to chelate coagulants and, in yeast, mannose is known as a competitive inhibitor of calcium-induced flocculation [26,27]. Future studies that focus on such extracellular saccharides as flocculation inhibitors should be informed by the fact that these inhibitors—if they be present—are likely growth-related and not maintenance-related products. This is clear from the data in Fig. 3, which demonstrate that spent nitrogen-free medium does not display the flocculation inhibition that spent nitrogen-containing medium does.

Study of water reuse

Ideally, water recovered from a flocculation process could be recycled for growing more cells. One potential problem with water recycling is that residual flocculant can cause flocculation in the growth vessel [14]. Despite its being a freshwater algae, *C. reinhardtii* is halophilic and can tolerate sodium chloride concentrations approaching 200 mM [28]. While we knew from experience that the 15 mM CaCl_2 concentration used in this study would not dramatically affect cell growth, we wanted to test whether it would induce unwanted flocculation of growth-phase cells.

The effect of calcium on the flocculation of growth-phase cw15 cells in fresh TAP media at pH 6.9 and 8.4, with and without 15 mM CaCl_2 , can be seen in Fig. 4. At the normal pH of a healthy culture (pH 6.9) no difference in flocculation is observed between cells grown with and without the added calcium ($p = 0.56$, $n = 4$). Likewise, no significant difference in flocculation is observed when the media pH is raised to 8.4 without the addition of calcium ($p = 0.78$, $n = 4$). However, when both calcium is added and the pH is raised, cells flocculate effectively (73% in 10 min, $p < 0.001$, $n=4$).

These results were obtained in fresh TAP media; reduced flocculation would likely be observed in spent growth media, as described previously. Nevertheless, it is clear that

cw15 cells will not flocculate because of the added calcium so long as a neutral pH is maintained. Thus, residual calcium should not be an obstacle to water recycling. Moreover, the 12e15 mM concentration of calcium used in the current study is similar to that found in seawater and, indeed, much higher concentrations can be found in wastewaters such as that found in landfill leachate [29], [30]. If such water were used as the influent process water for algae cultivation, it is possible that no addition of flocculant would be required downstream.

Conclusion

The wall-deficient cells of *C. reinhardtii*, *cw15*, flocculate readily from TAP medium with the addition of calcium chloride. This flocculation is enhanced by the addition of low concentrations (<5%) of methanol or ethanol. Equivalent flocculation results were achieved using 15 mM CaCl_2 or a combination of 12 mM CaCl_2 and 4.6% (m/v) methanol. A concentration of 8 mM CaCl_2 with 4.6% methanol induced over 70% flocculation in 10 min. Though the alcohol alone does not cause flocculation of *cw15*, it can be used as an easily recoverable substitute for a portion of the calcium used, thereby reducing the salination of process water. However, even if 15 mM CaCl_2 is used, results obtained here indicate that residual calcium in process water will not effect flocculation in recycled growth media such as that which has been observed elsewhere with chitosan flocculants [14].

From a flocculation perspective, we found that it is preferable to affect nitrogen starvation via media replacement rather than removing the nitrogen from the growth media by metabolic or other physical means. The reason is that an inhibitor (or enhancer) of flocculation is created by (or removed from) the medium as a result of cell growth. However, media replacement requires cell harvest. Thus, further work is needed to determine and eliminate the cause of diminished flocculation in growth media if flocculation is to be used for media replacement.

Additionally, future work will be required to determine the mechanism by which lower alcohols promote flocculation of *cw15* by calcium and whether higher concentrations of alcohol (>4.6%) might effectively replace more calcium. The effects of such variables as cell concentration, culture media types (particularly high salt), stir rates, temperature and pH should be explored, and the findings should be tested on other species of algae, particularly non-motile and wall-deficient ones.

Acknowledgments

Funding for this work was provided by the Marshall Foundation at The University of Arizona. We would also like to thank Dr. Lingling An for her statistical advice and Dr. Ursula Goodenough for her valuable insight.

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