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Cryopreservation of *Chlamydomonas reinhardtii*: A cause of low viability at high cell density ☆

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ABSTRACT

Cryopreservation is a practical method for stabilizing the genetic content of living algae over long periods of time. Yet, *Chlamydomonas reinhardtii*, the algal species most often utilized in studies requiring genetically defined strains, is difficult to cryopreserve with a consistently high post-thaw viability. Work described here demonstrates that *C. reinhardtii* retains high viability only when cryopreserved at a low cell density. Low viability at high cell density was caused by the release of an injurious substance into the culture medium. Rapid freezing and thawing under non-cryoprotective conditions released large amounts of the injurious substance. Heat denaturation of cells prevented the release of the injurious substance, but heating did not inactivate it after it was released. Even when concentrated, the injurious substance was non-toxic to cells under normal culture conditions. Reduced viability of cells cryopreserved in the presence of the injurious substance could not be attributed to changes in the tonicity of the medium. A mutant strain of *C. reinhardtii* (*cw10*) with a greatly diminished cell wall did not release a substance that reduced the post-thaw viability of wild-type or *cw10* cryopreserved cells. Cryopreservation of *cw10* cells was achieved with approximately the same post-thaw viability irrespective to the cell concentration at the time of freezing. Acid treatment of the injurious substance was able to partially diminish its injurious effect on cells during cryopreservation. We propose that diminished viability of *C. reinhardtii* cells cryopreserved at high cell densities is caused by the enzymatic release of a cell-wall component.

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Introduction

Chlamydomonas reinhardtii is a unicellular green alga that is broadly utilized in investigations of evolutionarily conserved cellular processes relevant to both animals and plants. Most notably, studies on *C. reinhardtii* have provided significant insights into how flagella/cilia and chloroplasts function [13]. *C. reinhardtii* provides an advantage over other biological systems because of its short generation time and relative ease of culturing. The development of molecular tools such as a completed genome sequence [18], a well-characterized genetic map [12], and the ability to transform all three (nuclear, chloroplast, and mitochondrial) genomes [13] have further established *C. reinhardtii* as a premier model system.

Over 2500 strains of *C. reinhardtii* and related species are maintained in the *Chlamydomonas* Resource Center at the University of Minnesota (Elizabeth Harris, personal communication). Thousands of additional strains have been generated and are maintained in smaller collections worldwide. Cultures are typically maintained using serial transfers, which are expensive and labor intensive. Many mutant strains appear identical morphologically to wild type, and some strains are especially prone to contamination. Vegetative cultures are susceptible to genetic reversion or other genetic changes. For example, strains expressing interference RNA constructs, which are used to selectively silence genes, have a high suppression rate and can be difficult to maintain in culture for even a few days. For these reasons, cryopreservation will become an increasingly important method for maintaining cultures of *C. reinhardtii*.

The first reports of *C. reinhardtii* cryopreservation demonstrated low (<10%) viability subsequent to thawing [9,17]. Later experiments have shown that higher viability can be achieved using methanol (MeOH) as a cryoprotective agent (CPA) in a two-step freezing process [3,19]. A viability of approximately 50% was reported using 2–10% (v/v) MeOH as a CPA and a cooling rate of approximately 1 °C per min down to a temperature of $\leqslant -55$ °C before plunging into liquid nitrogen [3].

In this study, we demonstrate that cultures of *C. reinhardtii* retain high viability only when cryopreserved at relatively low cell densities ($<2.5 \times 10^6$ cells ml^{-1}). Reduced post-thaw viability at higher cell densities is not caused by the physical proximity of cells, but rather the release of an injurious substance into the

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culture medium. Several lines of evidence suggest that the injurious substance is likely a component of the cell wall.

Materials and methods

Algal strains and culture conditions

Wild-type *C. reinhardtii* (UTEX 89) was obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX). *C. reinhardtii cw10* (CC-849), obtained from the *Chlamydomonas* Resource Center, was a gift from David Herrin. All cultures were maintained axenically on 1.5% agar slants of Tris–Acetate–Phosphate (TAP) growth media [6]. The slants were maintained at 22 °C under a 40-W cool white fluorescence lamp with a 16:8-h light:dark diurnal cycle. In preparation for cryopreservation experiments, liquid cultures were grown axenically in TAP medium at 22 °C in an illuminated incubator-shaker (Innova model 4340, New Brunswick Scientific, Edison, NJ) using a gyration rate of 100 cycles·min^{-1}. Overhead illumination was supplied by three 40-W cool white fluorescence lamps at an intensity of 90 μmol photons·m^{-2}·s^{-1} at the surface of the culture vessel, using a 12:12-h light:dark cycle. Liquid cultures were harvested for experiments prior to their reaching stationary phase ($<1.5 \times 10^7$ cells ml^{-1}).

Cell density and packed cell volume determination

A hemacytometer counting chamber (Hausser Scientific Company, Horsham, PA) was used in some experiments to determine the cell density of a culture, as described in Harris [8]. Another less direct method used a UV-visible spectrophotometer (Du 530, Beckman Coulter, Inc., Fullerton, CA) to measure the absorbance of an algal culture at 678 nm. For these measurements, approximately 2 ml of an algal culture was placed in a 1 cm cuvette fitted with a 3 mm thick translucent light scattering plate, and the optical density at 678 nm (OD_{678}) was recorded. A calibration curve was generated by relating the OD_{678} of a cell culture to the actual cell density as measured by the hemacytometer. The value obtained from OD_{678} measurements could then be used with the calibration curve to determine the cell density quickly. Separate calibration curves were generated for the wild-type and *cw10 C. reinhardtii* strains.

The relationship of packed cell volume (PCV) to culture density was determined in a culture at a cell density of 2×10^7 cells ml^{-1}. A 4-ml aliquot of culture was placed in a cytocrit tube, which was then subjected to centrifugation at 500*g* for 10 min. The ratio of the PCV to the total volume of the culture was determined. Packed cell volume (cytocrit) is proportional to cell density with 2×10^7 cells ml^{-1} equivalent to a PCV of 38% (v/v) of culture medium.

Cyropreservation procedure

In some experiments cultures were concentrated prior to cryopreservation by centrifugation at 1100*g* for 10 min at 25 °C, then suspending the pellet in fresh growth medium to the desired culture density. In all cryopreservation experiments a 2-ml cryovial (model 5000-0020, Nalgene Nunc International, Rochester, NY) contained 1.8 ml total volume of cryopreservation cocktail at room temperature. The cocktail included the alga strain to be cryopreserved at the specified final culture density in culture medium, 5% (v/v) MeOH (from a 20% stock solution) and other additions as indicated for individual experiments. Cultures were kept in subdued light whenever they were exposed to MeOH since bright light damages photosynthetic electron transport in the presence of methanol (J. Brand unpublished observation). Replicate cryovials were placed into a pre-chilled (4 °C) freezing canister (model 5100-0001, Nalgene), which was then placed in a −80 °C freezer. This system cooled the contents of the cryovials at a rate of approximately −1°C min^{-1} [3]. After 90 min, when cultures had reached −55 °C, cryovials were removed from the canister and placed into the vapor phase above liquid nitrogen (approximately −170 °C) in a storage dewar. They remained in the dewar for at least 2 h before thawing, by which time the contents of cryovials was approximately −170 °C. Cultures were thawed rapidly by transferring cryovials from the liquid nitrogen dewar to a 35 °C water bath for 5 min, which brought the contents of cryovials to approximately 20 °C. The thawed cultures were subjected to centrifugation at 800*g* for 5 min at ambient temperature. The supernatant was discarded and the pellet was suspended in 1.0 ml of fresh culture medium. This cell suspension was left undisturbed in darkness for at least 30 min prior to determining viability.

Viability

Viability of cells was determined by exploiting the observation that only living cells exclude Evan's Blue dye [5], as demonstrated for *Chlamydomonas* by Bodas et al. [1]. For experiments described here, 0.1% (w/v) Evan's Blue dye in double-distilled H_2O (ddH_2O) was combined with an equal volume of cell culture. After approximately 2 min of incubation, a portion of this mixture was placed on a hemacytometer and observed using a Zeiss (Thornwood, NY) bright-field microscope at 250X total magnification. *Chlamydomonas* cells retained their normal yellow–green color, while dead cells did not exclude the dye and appeared blue. Studies using wild-type *C. reinhardtii* demonstrated that viability as measured by the Evan's Blue exclusion method correlates well with viability as measured by the number of colonies formed on agar after quantitative plating. Strain *cw10* does not contain a cell wall and cells that do not survive cryopreservation lyse immediately after thawing. Viability of the *cw10* strain was determined by comparing the cell density of a culture before and immediately after cryopreservation using a hemacytometer counting chamber (Hausser Scientific Company).

Preparation of cell extracts and measurements of osomotic pressure

Cultures were concentrated by centrifugation at 1100*g* for 10 min. The pelleted cells were suspended to the desired concentration in fresh growth medium (or in ddH_2O when the culture was to be further concentrated by lyophilization). Concentrated 20-ml volumes of culture were rapidly frozen in a plastic bottle by placing the bottle of culture directly into a −80 °C freezer and leaving for at least 2 h. The frozen cultures were then rapidly thawed in a 25 °C water bath. This procedure killed and disrupted all cells in the culture as observed microscopically. The dead culture was subjected to centrifugation at 30,000*g* for 10 min at 4 °C to remove cellular debris, and the supernatant fraction was recovered as soluble cell extract. For heat-denaturing whole cells or cell extracts, a 10-ml volume of cell material was placed into a 20-ml test tube at ambient temperature. The test tube was closed loosely and placed for 5 min into a water bath that had previously been heated to 95–98 °C. The contents of the test tube were then cooled and subjected to centrifugation at 30,000*g* for 10 min at 4 °C in order to pellet and remove insoluble debris. In some experiments, supernatants in ddH_2O were concentrated to a dry powder by lyophilization (model 10-020, VirTis, Gardiner, NY) and then suspended in fresh growth medium to the desired concentration. Osmotic strengths of various sugars, amino acids and cell extracts in growth medium were measured with an Osmette A (model 5002,

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Precision Systems, Natick, MA) freezing-point depression osmometer.

Results

The post-thaw viability of cryopreserved *Chlamydomonas reinhardtii* decreases as the cell density of cryopreserved cultures increases. Cultures frozen at 1×10^6 cells ml^{-1} (PCV ~2%) retained approximately 35% viability while cultures frozen at 2×10^7 cells ml^{-1} (PCV ~38%) had very low (~1%) survival (Fig. 1). Cultures cryopreserved at cell densities of 2×10^6 cells ml^{-1} or lower typically retained viabilities higher than 50% when cells were diluted for cryopreservation without centrifugation. Viabilities were somewhat lower for the experiment shown in Fig. 1 because concentrating cells by centrifugation prior to cryopreservation decreases the post-thaw viability, typically by $\leqslant$15% (compare data presented in Figs. 1 and 2). The density of an actively growing culture prior to its preparation for cryopreservation did not have an appreciable effect on viability following cryopreservation (Fig. 2). Thus, the diminished viability of cryopreserved cultures at higher cell densities is an effect of culture density only after the cultures are prepared for cryopreservation.

A dilute culture of healthy cells was mixed with a high concentration of dead cells (killed by heating to 95 °C for 5 min), then cryopreserved. The viability of this mixed culture was nearly as high as viability of the same concentration of healthy cells without added dead cells (Fig. 3). This indicates that neither the physical proximity of dead cells, nor any substance released from heat-denatured cells, affected viability.

In contrast, the viability of a cryopreserved dilute culture of healthy cells was significantly diminished when cryopreserved in the presence of cell extract from a high concentration of dead cells that had been killed by rapidly freezing and thawing them in the absence of any CPA (Fig. 3). This indicates that low post-thaw viability of cryopreserved dense cultures is caused by a substance released into the culture medium during cryopreservation.

A confirmatory experiment was conducted by first cryopreserving replicate cultures at 3×10^7 cells ml^{-1}, which are conditions that have previously been shown to kill >99% of cryopreserved cells (Fig. 1). Upon thawing, cells were pelleted by centrifugation and the supernatant was collected from each culture. In preparation for a subsequent cryopreservation experiment, various concentrations of this supernatant were then separately added to identical dilute concentrations of healthy cells (Fig. 4). The culture concentration depicted for each sample in Fig. 4 corresponds to the addition of a supernatant volume equivalent to the specific culture concentration during the previous freeze/thaw. Cultures were

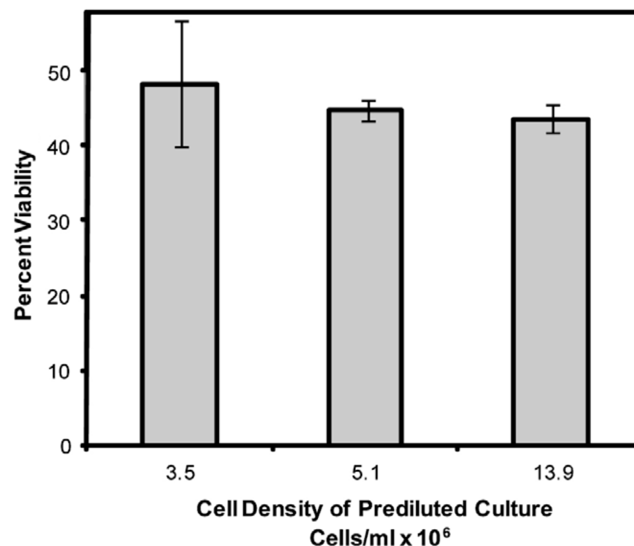

Fig. 2. Effect of the culture density of an actively growing culture prior to cryopreservation on the post-thaw viability of cells cryopreserved at the same culture density. Individual cultures of *C. reinhardtii* were grown to the concentrations shown, then each culture was diluted to a culture density of 1×10^6 cells ml^{-1} in fresh medium (without centrifugation) and cryopreserved. Error bars represent the results of duplicate experiments where viability of $n > 100$ cells was determined for each individual experiment.

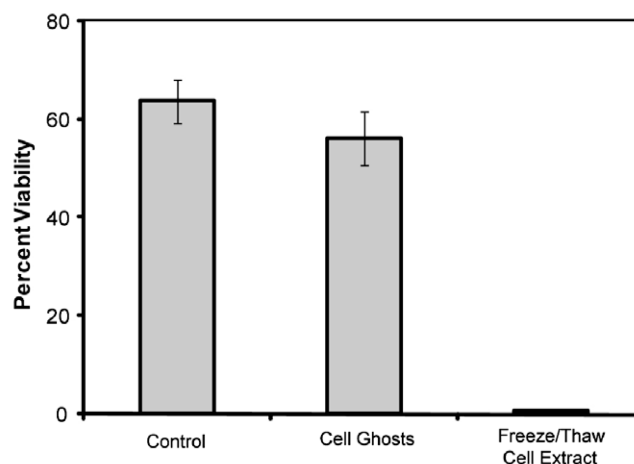

Fig. 3. Effect of cell ghosts produced by heat denaturation and of cell extracts prepared by rapidly freezing and thawing a culture on the viability of dilute cryopreserved cultures of *C. reinhardtii*. A healthy culture was concentrated to 3×10^7 cells ml^{-1} in fresh culture medium. For preparing dead cell ghosts, a portion of this culture was heated at >95 °C for 5 min. For preparing soluble cell extract, a portion of the same dense culture was rapidly frozen and thawed twice, then subjected to centrifugation to pellet the dead cells and recover the clear supernatant. In preparation for cryopreservation, 1.1-ml volumes of cell ghosts or extract (corresponding to 3.3×10^7 cells) were added to a healthy culture diluted to 1×10^6 cells ml^{-1} (without centrifugation). A control culture was prepared using the same dilute concentration of healthy cells without any additional cell components. All cultures were then cryopreserved and thawed, and their viabilities were determined. Error bars represent the results of duplicate experiments where viability of $n > 100$ cells was determined for each individual experiment.

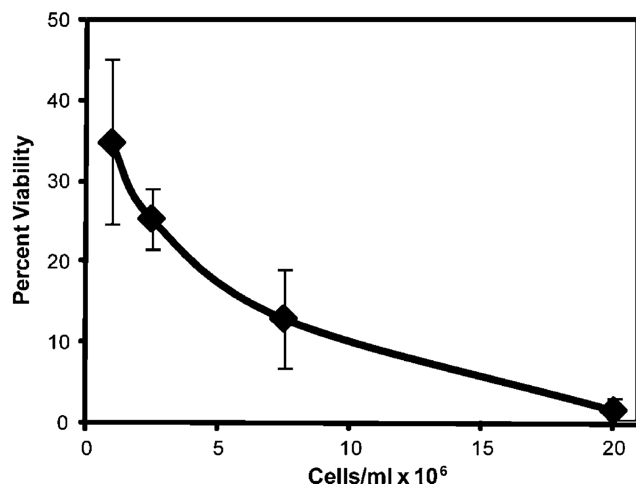

Fig. 1. Effect of cell density at the time of freezing on viability of *C. reinhardtii* cells subsequent to cryopreservation. A rapidly growing culture at a density of 1.6×10^6 cells ml^{-1} was pelleted by centrifugation, and suspended to a density of 3×10^7 cells ml^{-1} in fresh culture medium. Aliquots of this dense culture were then diluted to the concentrations shown. All cultures were subsequently cryopreserved and thawed prior to determining their viability. Error bars represent the results of duplicate experiments where viability of $n > 100$ cells was determined for each individual experiment.

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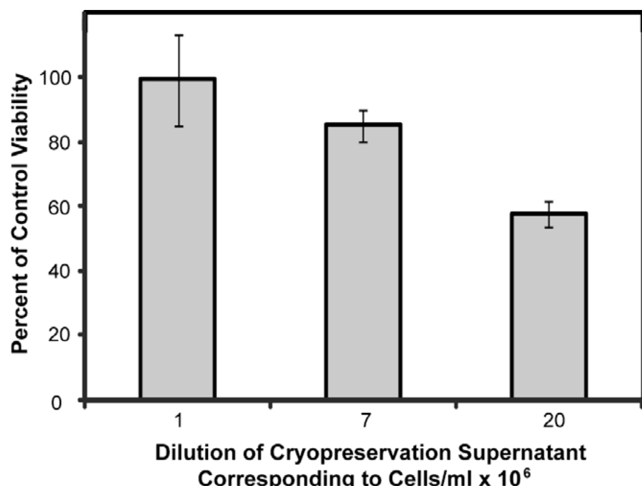

Fig. 4. Effect of supernatant obtained from cells cryopreserved at a high cell density on the viability of dilute cryopreserved *C. reinhardtii* cells. Replicate healthy cultures were cryopreserved at 3×10^7 cells ml^{-1} as described in materials and methods. Upon thawing, cells were pelleted by centrifugation and the supernatants were collected and combined. The combined supernatant was diluted to volumes corresponding to cells at the concentrations shown. The diluted supernatants were then added to dilute healthy cultures at 1×10^6 cells ml^{-1}. A control culture at 1×10^6 cells ml^{-1} was cryopreserved without any added supernatant. Cultures were cryopreserved and their viabilities were determined subsequent to thawing. Data are all expressed as percent of the viability of the control culture. Error bars represent the results of triplicate experiments where viability of $n > 100$ cells was determined for each individual experiment.

cryopreserved, thawed, and their viabilities determined. Post-thaw culture viability was inversely proportional to the amount of substance added to the cells (Fig. 4). Cultures cryopreserved with a concentration of supernatant equivalent to 1×10^6 cells ml^{-1} retained a post-thaw viability that was virtually identical to that of the untreated control. However, cultures cryopreserved with volumes of supernatant equivalent to 7×10^6 and 2×10^7 cells ml^{-1} had post-thaw viabilities that were diminished by approximately 15% and 42% of the control, respectively. Thus, the cryopreserved extract contains an injurious substance that is released into the medium and affects cells in a dose-dependent manner.

The reduction in post-thaw viability resulting from substance release into the medium during cryopreservation was not caused by an increase in tonicity (Table 1). A concentration of cell extract that caused an almost a complete loss of post-thaw viability, when added to a dilute culture of *C. reinhardtii* prior to cryopreservation, did not appreciably increase the osmotic strength of the medium. A 10-fold higher concentration of extract, which completely precluded the survivability of a culture subsequent to cryopreservation, only increased the osmotic strength by approximately 15%. In contrast, a 0.01 M concentration of a sugar or an amino acid, which also increased the osmotic strength of the medium by 15%, had no injurious effect on the viability of dilute cryopreserved cultures. A much higher concentration (0.15 M) of a sugar or an amino acid, which increased the osmotic strength of the medium over 3-fold, precluded viability of cryopreserved cultures, demonstrating that very high osmotic strengths should be avoided during cryopreservation. The osmotic strengths of solutions shown in Table 1 were virtually identical with or without the addition of 5% MeOH and living cells (data not shown).

The supernatant from a dense culture that was heated to 95 °C prior to rapid freezing and thawing in the absence of CPA only partially suppressed viability of cryopreserved dilute cultures of *C. reinhardtii*. Yet, heating the injurious supernatant to 95 °C following the rapid freeze thaw procedure used to generate it did not diminish the injurious effect of the supernatant on cryopreserved cultures, as determined by their low post-thaw viability (Fig. 5). Thus, an event required to release the injurious substance is apparently heat-labile, although the injurious substance is not readily denatured by heat after it is released.

To rule out the possibility that a component toxic to cells under normal growth conditions is being liberated from cells during freezing and thawing, a dilute (1×10^6 cells ml^{-1}) healthy culture containing the injurious substance from an extract of rapidly frozen and thawed cells was placed under normal culture conditions and its rate of growth over a six-day period was compared to that of an equivalent culture incubated without the injurious substance. Both cultures grew vigorously, although growth was slightly faster in cultures devoid of the extract (Fig. 6).

To investigate whether the substance that diminishes the viability of cryopreserved cultures is a component of the cell wall, a culture of a mutant strain of *C. reinhardtii* (*cw10*) with a greatly reduced cell wall [4,10] was killed by rapid freezing and thawing in the absence of any CPA. Soluble cell extract was prepared from a frozen and thawed *cw10* culture by recovering the supernatant from high-speed centrifugation. Dilute healthy cultures of both wild-type and *cw10* cells were cryopreserved in the presence of this extract. Fig. 7 demonstrates that, in contrast to extract from wild-type *C. reinhardtii*, concentrated extract from *cw10* does not diminish the viability of either wild-type or *cw10 C. reinhardtii* cells.

To further substantiate that *cw10* cells did not release an injurious substance into the culture medium and that the physical proximity of cells did not hinder the ability of a culture to survive cryopreservation, an actively growing *cw10* culture was cryopreserved at several cell densities and its viability was compared to that of wild-type cells at the same density (Fig. 8). *C. reinhardtii cw10* cells are more fragile than are wild-type cells. Thus, many fewer *cw10* cells were observed after thawing from cryopreservation than were initially frozen. However, virtually all of the remaining intact cells excluded Evan's Blue dye. Therefore, viability of *cw10* could be determined by comparing the cell density of a culture before and immediately after cryopreservation. Morris et al. [19] made a similar observation with *C. reinhardtii cw15*, an allele to *cw10* [4]. The results shown in Fig. 8 indicate that viability of *cw10* remained high regardless of the culture density during cryopreservation. Wild-type cultures retained slightly higher viabilities than did *cw10* when cryopreserved at low culture densities, but *cw10* cells retained much higher viabilities than did wild-type cells when cryopreserved at high culture densities.

Table 1
Osmotic effect of various solutions on viability of cryopreserved *C. reinhardtii*.

Substance added to cell culture	Osmolality (mOs kg^{-1})	Average percent viability
Culture medium only	59	52.5
Injurious cell extract (1x)	60	0.5
Injurious cell extract (10x)	68	0.0
0.01 M sugar or amino acid	69	55.4
0.15 M sugar or amino acid	~189	0.0

Osmotic strengths of various compounds or injurious cell extracts were measured. Dilute cultures (1×10^6 cells ml^{-1}) were then separately cryopreserved with the shown osmolalities of these substances, and viability was determined after thawing. Cell extracts were prepared from two separate cultures that were first concentrated to 1×10^8 cells ml^{-1}, one in ddH_2O and the other in TAP medium. Both cultures were rapidly frozen and thawed twice, and subjected to high-speed centrifugation in order to remove cellular debris. The supernatant in TAP medium was used directly as 1x cell extract. The supernatant in ddH_2O was lyophilized, and then dissolved in TAP medium to 1/10 of the original supernatant volume (10x cell extract). Two sugars (L(+)-arabinose and D(+)-galactose), and two amino acids (L-proline and 4-hydroxy-L-proline), were tested at concentrations shown. Average percent viabilities are the average of duplicate experiments where viability of $n > 100$ cells was determined for each individual experiment. Details of these procedures are described in materials and methods.

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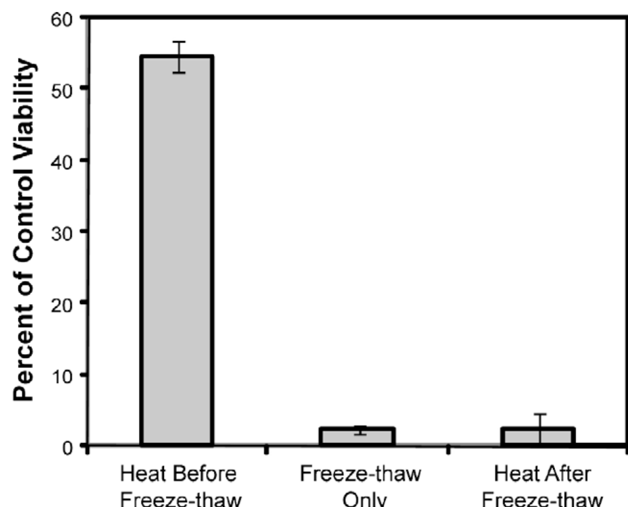

Fig. 5. Effect of the cell extract from a rapidly frozen and thawed culture on the viability of a dilute cryopreserved culture when the frozen/thawed culture was heat-denatured before or after rapid freezing and thawing. An actively growing *C. reinhardtii* culture was concentrated to 2×10^7 cells ml^{-1} in fresh culture medium. A portion of this culture was placed in a hot water bath to bring its temperature to ~95 °C for 5 min in order to denature heat-labile substances, and then was rapidly frozen and thawed twice. A separate portion of the concentrated culture was rapidly frozen and thawed twice before it was placed in the ~95 °C water bath for 5 min. A third portion of the culture was rapidly frozen and thawed twice, but not heated. Each treated culture was subjected to centrifugation to remove cellular debris, and a 0.9-ml volume of each supernatant (corresponding to extract from 1.8×10^7 cells) was added to a healthy culture diluted to 1×10^6 cells ml^{-1} (without centrifugation). A control culture was prepared identically, except in the absence of supernatant fraction. All cultures were then cryopreserved and thawed, and their viabilities were determined. Data are expressed as percent of the viability of the control culture. Error bars represent the results of duplicate experiments where viability of $n > 100$ cells was determined for each individual experiment.

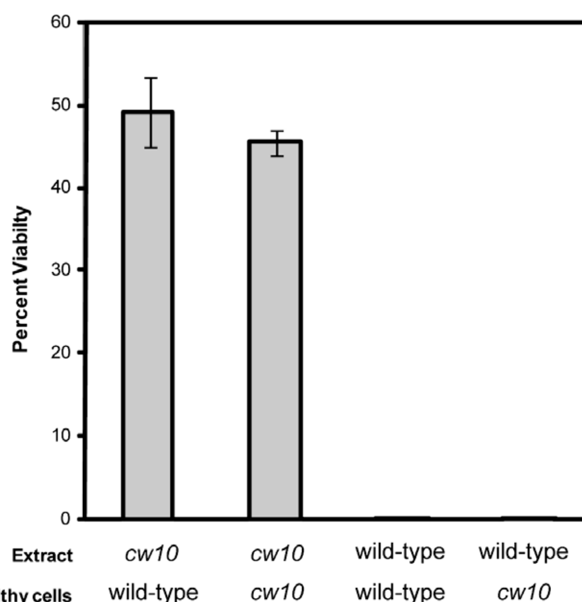

Fig. 7. Effect of extracts produced from rapidly frozen and thawed *C. reinhardtii cw10* mutant or wild-type cultures on viability of cryopreserved healthy *C. reinhardtii* wild-type and *cw10* cultures. Actively growing *cw10* or wild-type cells were concentrated to 1×10^8 cells ml^{-1} in ddH_2O, rapidly frozen and thawed, and subjected to centrifugation to remove cellular debris. The supernatant was lyophilized and then dissolved TAP medium to 1/10 of the original supernatant volume (10x cell extract). A 0.2-ml volume of each concentrated extract (corresponding to 2×10^8 cells) was added to separate healthy cultures of wild-type and *cw10* cells diluted to 1×10^6 cells ml^{-1} (without centrifugation). All cultures were then cryopreserved and thawed, and their viabilities were determined. Error bars represent the results of duplicate experiments where viability of $n > 100$ cells was determined for each individual experiment.

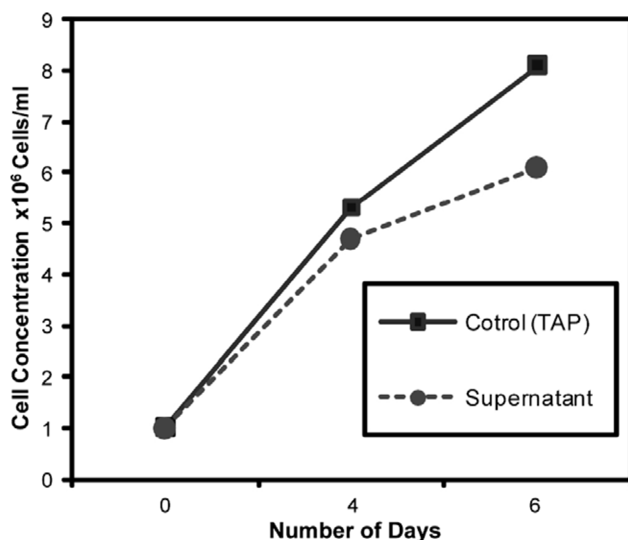

Fig. 6. Effect of concentrated injurious substance on an actively growing culture of wild-type *C. reinhardtii*. A healthy culture was concentrated to 3.4×10^8 ml^{-1} and killed by rapidly freezing and thawing in the absence of CPA. Dead cell particulate matter was removed by centrifugation, and the soluble cell extract was recovered. A healthy culture was then diluted to 1×10^6 cells ml^{-1} in 1.8 ml of the recovered extract (corresponding to 6.1×10^8 cells). A control culture was prepared identically, except it was diluted in fresh culture medium instead of the soluble cell extract. Both cultures were placed under normal culturing conditions, and densities were measured after four and six days of growth.

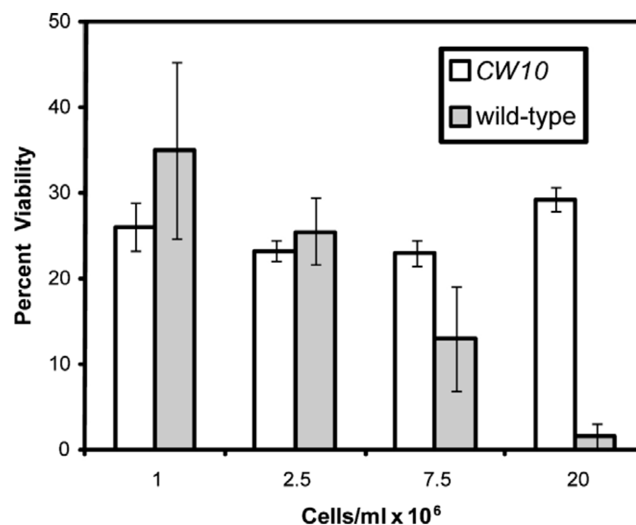

Fig. 8. Effect of cell density of *C. reinhardtii* wild-type and *cw10* mutant cultures at the time of freezing on viability after cryopreservation. Actively growing cultures of wild-type and *cw10* cells were each concentrated to 3×10^7 cells ml^{-1} in fresh growth medium. Aliquots were diluted in fresh culture medium to the concentrations shown. All cultures were then cryopreserved and thawed, and their viabilities were determined. Error bars represent the results of duplicate experiments where viability of $n > 100$ cells was determined for each individual experiment.

We determined if exposure of the injurious substance to low pH at a temperature near boiling diminished its potency, since this procedure hydrolyses glycosidic bonds. Because viability is diminished in cultures cryopreserved at high osmolalities (Table 1), injurious cell extract was prepared from a dense culture of cells rapidly frozen and thawed under non-cryprotective conditions in growth

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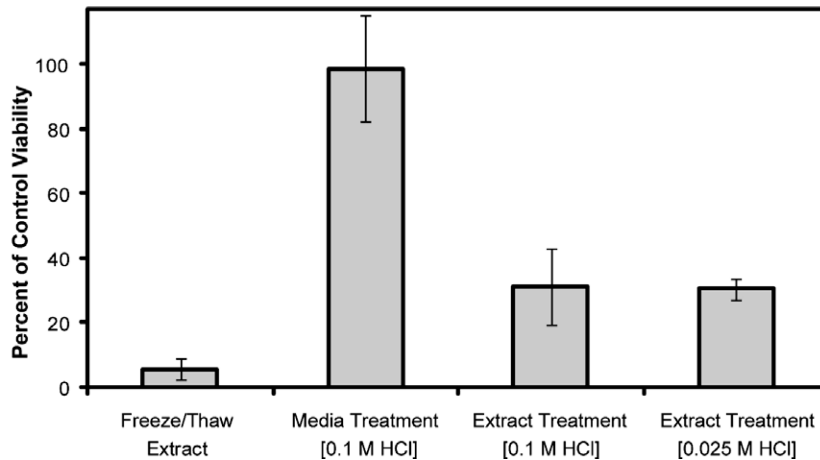

Fig. 9. Effect of acid treatment on the potency of the injurious cell extract. Actively growing wild-type cells were concentrated to 3×10^7 cells ml^{-1} in ¼ growth media, rapidly frozen and thawed, and subjected to centrifugation to remove cellular debris. Concentrated HCl was used to dilute separate portions of the injurious extract to final concentrations of 0.025 M and 0.1 M HCl, which brought the pH of the solutions to approximately 2.1 and 1.5, respectively. Concentrated HCl was used to dilute a ¼ growth media control solution to a final concentration of 0.1 M HCl, which had a pH of approximately 1.5. All three solutions were placed in a boiling water bath for 30 min. The solutions were cooled to room temperature and then brought to pH ~7.0 by the addition of concentrated NaOH. In preparation for cryopreservation, an equal volume of each solution was added to a separate dilute concentration of cells. For comparison, identical volumes of untreated growth medium and untreated injurious cell extract were added to a dilute cell cultures. The cultures were cryopreserved and their viabilities were determined subsequent to thawing. Data are expressed as percent of the viability of the control culture. Error bars represent the results of triplicate experiments where viability of $n > 100$ cells was determined for each individual experiment.

medium diluted to ¼ its normal concentration with ddH_2O. Concentrated HCl was used to dilute aliquots of the injurious extract to final concentrations of 0.025 M and 0.1 M HCl, respectively. For comparison, diluted culture medium without the injurious substance was exposed to acid treatment. All three treated solutions were placed in a boiling water bath for 30 min, and then neutralized by the addition of concentrated NaOH. In preparation for cryopreservation, an aliquot of each solution was added to a dilute suspension of cells. As controls, untreated growth medium and untreated injurious cell extract were added to dilute suspensions of cells. Cultures were cryopreserved and their viabilities were determined subsequent to thawing (Fig. 9). Cultures cryopreserved in the presence of acid-treated extracts retained significantly higher viabilities than when cryopreserved in the presence of untreated injurious substance, but viabilities were only 30% of untreated controls. Viabilities were virtually identical in cultures cryopreserved in the presence of injurious substance subjected to a 4-fold range of H_3O^+ concentration. The culture cryopreserved in solution containing acid treated growth medium in the absence of injurious substance retained viability nearly as high as in the untreated control, indicating that the increase in osmolality during acid treatment and subsequent neutralization did not affect viability.

Discussion

Algal cryopreservation procedures generally do not prescribe a specific culture density during freezing. In fact, cryopreservation studies of microorganisms generally neglect this variable. Consequently, the significance of culture density during cryopreservation in most species is unknown.

Mammalian erythrocytes are readily hemolysed when cryopreserved at high cell densities [20]. Several explanations of this phenomenon have been proposed. Mazur and Cole [14,15] have suggested that the close proximity of erythrocytes in dense suspension causes pronounced hemolysis due to extensive cell-cell and cell-ice contact. Other studies [21,22] indicate that the tonicity of the suspension medium relative to the osmolality of red blood cells induces cellular damage during cryopreservation. Alternatively, Wagner et al. [26] proposed that a "biochemical" phenomenon is at least partially responsible for red blood cell hemolysis at high cell densities. No specific compound was identified, nor was any specific mechanism proposed.

Data presented here indicate that a close physical proximity of *C. reinhardtii* cells during cryopreservation at higher cell densities is not a direct cause of the post-thaw reduction in viability. Neither can cell damage during cryopreservation be attributed to the tonicity of the medium. Instead, one or more specific substances apparently are released from the cells into the medium sometime during the freezing and/or thawing process, thereby decreasing the post-thaw viability of a culture. The injurious substance (or substances) described here is likely released from cell walls through an enzymatic process. This supposition is supported because (i) heating precludes the release of the substance, (ii) heating does not diminish the activity of the substance once it has been generated, (iii) a cell-wall-deficient strain does not generate the injurious substance, and (iv) a similar post-thaw viability is achieved in a cell-wall deficient strain irrespective to the concentration of cells during cryopreservation. The mechanisms causing diminished viability in *C. reinhardtti* may be different than in red blood cells because erythrocytes contain only a thin (2.0–4.0 nm) glycocalyx external to the plasma membrane [7], while *C. reinhardtii* has an extensive (>80 nm) multi-layered wall structure [23].

In *C. reinhardtii*, two distinct cell-wall digestive enzymes have been characterized including vegetative cell autolysin and gamete wall autolysin [8]. Vegetative cell autolysin releases cells from their mother cell wall after mitosis [24,25], but is not capable of degrading the walls of interphase cells such as those used in this study [24]. Gamete wall autolysin is typically released from cells during mating [2] and is only solubilized from frozen and thawed cells following significant mechanical shearing [16]. Because vegetative cell autolysin acts only on the mother cell wall of freshly divided cells, and gamete wall autolysin is not biologically available to vegetative cells upon freezing and thawing alone, it seems unlikely that the injurious substance released from cell walls during cryopreservation is produced by either of these enzymes. However, our data do not exclude the possibility that the substance responsible for diminished viability during cryopreservation is catalyzed by another enzyme.

Preliminary data using gel exclusion chromatography indicate that the injurious substance(s) released by cells into the culture medium during freezing and thawing is a small (MW <1500 dal-

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tons), water-soluble, heat stable, organic compound (Piasecki and Brand unpublished). Acid treatment of the injurious substance using various concentrations of HCl was able to consistently reduce the potency of the injurious extract by approximately 30%. Although extraction of the cell wall of wild-type *C. reinhardtii* with ethanol and diethyl ether releases approximately equal amounts of carbohydrate, protein, and inorganic matter (ash), a small amount of water-soluble carbohydrate is released from the walls of physically disrupted cells without chemical treatment [11]. This fraction contains rhamnose, arabinose, mannose, galactose, and glucose. The physical stress induced by cryopreservation might release this same water-soluble carbohydrate, although our results show that neither arabinose nor galactose at relatively high concentration (10 mM) inhibits viability of cryopreserved *C. reinhardtii* (Table 1), nor does mannose at the same concentration (data not shown). Our data are consistent with an injurious effect of a water-soluble component of the cell wall.

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