Customized cryopreservation protocols for chlorophytes based on cell morphology

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ABSTRACT

The development of efficient algae cryopreservation methods is pivotal to the establishment of long-term culture collections as well as algae breeding and genetic modification programs. However, the unpredictable responses of distinct algal species to cryopreservation agents and protocols have hampered the standardization of universal methods so far. The results presented in this study indicate that intrinsic biological factors (i.e.: cell morphology and phylogenetic origin) play a role in the definition of which culture age and cryoprotectant agent type and concentration should be used to achieve successful cryopreservation. Through the use of Central Composite Rotatable Design (CCRD) it was possible to define optimized protocols for cryopreserving three major morphotypic groups found among chlorophytes (i.e.: coenobium, coccoid or palmella forming strains). These optimized protocols were then validated upon fifteen strains from Sphaeropleales, Chlamydomonadales and Chlorelalles clades. Analysis reveals that the use of DMSO as the sole cryoprotectant provided the highest post-freezing cell viability recovery rates for 80% of the coccoid strains tested, while the combination of glycerol and PEG400 allowed efficient cryopreservation of 100% of the coenobium and palmella forming strains evaluated. In addition, results suggest that coenobium-forming strains have higher resistance to freeze/thawing when frozen at mid-log phase of growth, while coccoid and plamelloid strains present higher survival rates when cryopreserved at late-log phase. Such information is of valuable practical use since a simple visual inspection of algae strain's predominant morphotype can guide the choice of the most suitable cryopreservation protocol.

1. Introduction

There is a growing interest in using microalgae for biotechnological applications such as the production of biofuels, special oils, pigments, polymers, animal feed and other bioproducts [1-4]. The green algae taxon, Chlorophyta, comprise approximately 8000 described species of single-celled or colonial photosynthetic organisms that are naturally present in different aquatic and humid terrestrial ecosystems [5,6]. This largely unexplored genetic diversity provides a major reservoir for microalgae breeding programs. Pivotal to this task though, is the prior establishment of long-term culture collections. Traditional algae culture maintenance methods based on continuous subculturing in liquid or solid media, however, are time-consuming, expensive and prone to genetic drift and contaminations [7]. Therefore, procedures that can provide long-term storage of microalgae, such as cryopreservation, are the most suitable alternatives [8]. Indeed, low temperatures inhibit chemical and physical processes and can maintain biological samples in a metabolic inactive state for long periods of time [9]. However, it has been reported that procedural steps, such as cooling rate, the type and concentration of the cryoprotectant agent used and post-thawing steps, as well as biological features, such as culture age, cell size and morphology, can greatly influence the levels of cell viability recovery. This occurs due to the distinct responses of complex biological systems to cell damage caused by water loss and ice crystals formation during the freezing process [7,10-12].

Relative success of the standardization of procedural steps such as cell concentration, cooling rate and thawing protocols for a broad range of algal species has been reported [8]. On the other hand, a universal type and concentration of cryoprotectant agent (CPA) has not been achieved so far [7,9,11,13-15]. CPAs promote cell viability by acting...
both extracellular and intracellularly. For example, cell permeable cryoprotectants, such as dimethyl sulfoxide (DMSO), glycerol and me-thanol, disrupt ice crystal formation intracellularly, while non-perme-able agents, such as polyethylene glycol (PEG), induce cell dehydration by promoting intracellular water efflux. Furthermore, synergies among combined CPAs have also been observed for the cryopreservation of some algal species when using double (sucrose + DMSO) or even triple (DMSO + proline + ethylene glycol) mixtures [7–9]. Therefore, each type of CPA (or CPAs mixture) appears to affect post-thawing cell vi-a-bility recovery differently according to the algal strain, suggesting that intrinsic biological factors play a role in the efficiency of each type of CPA. This study evaluated the correlation between biological factors (i.e.: cell morphology and phylogenetic origin) and the efficiency of distinct CPAs. The goal was to investigate whether strain morphology typing could provide insights about CPAs efficiency thereby allowing the development of cryopreservation protocols customized for major chlorophyte groups.

2. Material and methods

2.1. Microalgae strains

Microalgae strains Desmodesmus spinosus|LBA35, Chlorella sorokiniana|LBA39 and Chlamydomonas biconvexa|LBA40 were used in the selection of cryoprotectants agents and on the cryopreservation optimization experiments as representatives of three different mor-photypes (i.e.: colonial coenobial, unicellular coccoid, colonial pal-melliod) (Supplementary Fig. 1). Fifteen microalgae strains re-presentative of each morphotype were selected from the Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefineries at Embrapa (Brasilia/DF-Brazil) and the subsequent va-lidation of the optimized protocols was performed on all fifteen microalgae strains (Supplementary Table 1).

2.2. Microalgae cultivation

Microalgae were cultivated in erlenmeyers flasks (500 ml) with 300 ml of BG11 medium [16]. They were inoculated with optical density adjusted for 0.01 (O.D. 0.01, \( \lambda = 680 \) nm) and kept under artificial illumination of 100 \( \mu \text{E}m^{-2}s^{-1} \), 12/12 h light cycle, temperature of 30 °C, aeration of 10 L h\(^{-1} \), for a period of 10 days. Biomass growth was monitored through optical density (O.D.) readings at 680 nm.

2.3. Cryopreservation

Microalgae cell suspensions were adjusted (centrifuged and sus-pended in fresh medium) to optical density equal to 1 (O.D. = 1/ \( \lambda = 680 \) nm) immediately prior to cryopreservation for normalization purposes. Aliquots of the cultures (1.5 ml) were transferred to 2 ml polypropylene cryotubes. Cryoprotectants (Glycerol, DMSO and PEG 400) were then added (or not, in the case of control samples) at concentrations of 10% (cryoprotectants agents screening assays) or in concentrations ranging from 0 to 10%, in the case of the optimization of microalgae cryopreservation protocols assays. The cryotubes were subsequently placed in a freezing container (Mr. Frosty™ Freezing Container) which allows a cooling rate of approximately \(-1 \)°C min\(^{-1} \). The container was placed in ultra-freezer (\(-80 \) °C) and after 4 h the frozen cryotubes were removed from the container, placed in storage boxes and stored in ultra-freezer at \(-80 \) °C. After 15 days, the cryotubes were thawed by submersion in a 35 °C water bath for approximately 2 min. Samples were centrifuged for 5 min at 10,732 g and the supernatant discarded. The pellets were transferred to test tubes (20 x 150 mm) and suspended in 3.5 ml of fresh BG-11 medium. Cell viability was measured immediately (0 h) and also 24 h after thawing. For the measures taken 24 h after thawing, the tubes were maintained in growth chambers under illumination cycles of 12 h light/12 h dark and temperature of 25 °C for 24 h.

Results were statistically evaluated by analysis of variance (ANOVA), using the Tukey test at significance level of 5%, with the software Statistica [17].

2.4. Cell viability assay

Cell viability was determined using TTC (2, 3, 5 – Triphenyltetrazolium chloride) method. Traditionally, this method was used in the determination of cell viability in plants and seeds [18–20], but know is well consolidated also for algae studies [21–25]. The assay is based on the activity of dehydrogenase enzymes, which catalyze respiratory reactions in mitochondria. These enzymes reduce the TTC salt (colorless) to formazan in living cells. The resulting formazan crystals present red color and the color intensity is used to measure mitochondrial activity and, consequently, cell viability, since the reduc-tion of respiratory rates reflects the metabolic activity of the cells [20]. Cell viability was determined based on the absorbance (ABS) at 490 nm of algal strains samples submitted to TTC method. Absorbance was measured both before cryopreservation and after thawing. Cell viability was calculated as shown:

\[
\text{Cell Viability (\%)} = \frac{\text{Sample ABS 490nm (after thawing)}}{\text{Sample ABS 490nm (before cryopreservation)}} \times 100
\]

(1)

In test tubes, 1.5 ml of each culture was homogenized in 3 ml of TTC reagent 0.5% (w/v) and then incubated for 24 h in the dark at 28 °C. After this period, 6 ml of ethanol 95% (v/v) was added to the tubes which were then placed in boiling water for 10 min in order to release the colored formazan compound. The material was then centrifuged for 5 min at 10,732 g for solids separation and the supernatant was used for absorbance spectrophotometer readings at 490 nm. Cell viability was confirmed by monitoring algal growth (optical density of the cultures at 680 nm), up to 15 days after thawing (data not shown).

2.5. Cryoprotectant agents screening

An initial screening using either two cell penetrating CPAs, glycerol and DMSO, or one non-permeable CPA, polyethylene glycol 400 (PEG400), at 10% concentration was performed. Algal cells were frozen after 9 days of cultivation (stationary phase) and cell viability was measured immediately (0 h) after thawing and 24 h post-thawing. Results were statistically evaluated by analysis of variance (ANOVA), using the Tukey test at significance level of 5%, with the software Statistica [17].

2.6. Optimization of microalgae cryopreservation protocols

Cell viability after freezing/thawing was analyzed at three different algal growth phases: mid-log phase (3rd day of cultivation), late-log phase (6th day of cultivation) and stationary phase (9th day of cul-tivation). A Central Composite Rotatable Design (CCRD) was used to investigate the effect of cryoprotectants - concentration ranging from 0 to 10% (v/v) (independent variables - Glycerol and PEG400 for D. spinosus|LBA35 and C. biconvexa|LBA40 strains; DMSO and Glycerol for C. sorokiniana|LBA39 strain) on viable cells recovery after freezing (dependent variable). The CCRD approach was used because in this method variables are analyzed simultaneously, thus it is possible to verify and quantify synergistic and antagonistic effects. Moreover, CCRD method employs a reduced number of experiments or repetitions, considerably reducing human manipulation errors [26–28]. Therefore the experimental strategy was composed of a 2\(^2\) factorial design, five levels of each independent variable, 4 axial points and 3 repetitions of the central point, in a total of 11 experiments (Supplementary Table 2) for each algal morphotype at each one of the three growth phase studied.
The following polynomial equation was fitted to data:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1^2 + \beta_4 x_2^2 + \beta_5 x_1 x_2$$  

(2)

where $\beta_n$ are constant regression coefficients; $y$ is the response (cell viability %), and $x_1$ and $x_2$ are the coded independent variables. The analysis of variance (ANOVA), determination of $R^2$ and the generation of three-dimensional graphs were carried out using the software Statistica [17]. The experimental validation was performed through the comparison between the cell viability of samples frozen for 15 days and 6 months under optimized conditions against the cell viability obtained for samples frozen in the absence of cryoprotectants. Results were statistically evaluated at significance level of 5% through the interface Experimental Design (DOE) followed by analysis of variance (ANOVA), using the software Statistica [17].

3. Results and discussion

3.1. Cryoprotectant agents screening

The core hypothesis empirically investigated in this study is that intrinsic biological factors (i.e.: algal cell morphology and phylogenetic origin) are major determinants for the efficacy of distinct cryopreservation protocols. In order to address this issue, three distinct microalgae strains were initially tested: the coenobia forming strain *Desmodesmus spinosus* |LBA35, the unicellular coccoid strain *Chlorella sorokiniana* |LBA39 and the palmella forming strain *Chlamydomonas biconvexa* |LBA40 (Supplementary Fig. 1).

The results of the cryoprotectant agents screening is presented in Fig. 1. These two measurements (immediately after thawing (A) and 24 h post-thawing (B)) were used because they allow the quantification not only of algal resistance to freezing/thawing but also cell viability recovery, since some CPAs can be toxic to certain algal strains [7]. Quantification of algal cells viability after cryopreservation is often performed through plating and counting of colony forming units [8,9,29,30]. However, this method is laborious, time-consuming and known to be error prone [30,31]. Therefore, the method of choice for this study was based on the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to water-insoluble reddish formazan salts by enzymes of the respiratory chain, a measure of cell mitochondrial activity and viability of eukaryotic organisms that has been used for decades [20]. This method allows the rapid quantification of algal cells viability through simple spectrophotometric absorbance measurements.

Fig. 1 shows a higher cell viability recovery for strains *D. spinosus* |LBA35 and *C. biconvexa* |LBA40 after cryopreservation in the presence of glycerol and PEG400. In contrast, DMSO, promoted higher cryoprotectant effect for *C. sorokiniana* |LBA39 (Fig. 1B). It is important to highlight that algal strains were not only physiologically active but also capable of cell division as observed by visual inspection of algal cultures growth up to 15 days post-thawing (Data not shown). Representative pictures of algal growth recovery post-thawing, either before or after the addition of TTC, are shown (Supplementary Fig. 2).

The variable efficiency of CPAs according to algal strain was expected and has been reported previously [7,8,13,32]. The higher viability recovery obtained using glycerol and DMSO for the cryopreservation of *C. sorokiniana* |LBA39, however, contrasts with the results reported by Nakaniishi et al. [9], that did not observe significant cryoprotectant effect when using glycerol and DMSO upon *Chlorella* strains. It is important to highlight though, that the CPAs concentrations used in that study were lower (i.e. 2.5–5%) than the concentrations of 10% used in here. On the other hand, studies from Crutchfield et al. [33] and Yang & Li [34] reported low efficacy of DMSO as a CPA for *Chlamydomonas* strains, corroborating the results shown for *C. biconvexa* |LBA40 (Fig. 1).

3.2. Optimization of algal cryopreservation protocols

The effect of culture age in cryopreservation tolerance has been reported as variable among different algal strains [29,35–37]. For example, reference algae collections, such as UTEX Culture Collection of Algae, Culture Collection of Algae and Protozoa (CCAP) and Culture Collection of Autotrophic Organisms (CCALA), recommend cells to be frozen during log phase of growth. In contrast, however, increased resistance to cryopreservation has been observed for older cultures (stationary phase) of *Nannochloropsis gaditana*, *Chaetoceros gracilis*, *Tetraselmis chuii* and *Chlorella vulgaris* [29,30]. Furthermore, Osorio et al. [35], Piasecki et al. [36] and Salas-leiva & Dupré [37] reported higher cell viability recovery in experiments performed during late-log growth phase.

In order to investigate the influence of algal growth phase on cell viability recovery post-thawing, as well as to optimize cryoprotectant agents efficiency, *D. spinosus* |LBA35, *C. sorokiniana* |LBA39 and *C. biconvexa* |LBA40 strains were submitted to cryopreservation based on experimental planning using central composite rotatable design (CCRD) (Supplementary Table 2). The two most efficient CPAs previously screened for each strain (Fig. 1) were used upon cells derived from cultures in the following growth phases: mid-log phase (3rd day), late-log phase (6th day) and stationary phase (9th day) (Supplementary Fig. 3). The post-thawing cell viability results (Supplementary Table 3) were used to calculate regression coefficients and the determination coefficients ($R^2$) of the coded second order polynomial equations of each strain using cells frozen at the three distinct growth phases (Supplementary Table 4). It is possible to verify significant synergistic effects ($p < 0.05$) among the studied CPAs (Supplementary Table 4). After the elimination of non-significant factors (NS), the analysis of variance (ANOVA) and the F test were performed in order to verify the significance of the regressions obtained and possible lack of fit of the models (data not shown), at confidence level of 95%. It can be observed that strain *D. spinosus* |LBA35 presents highest post-freezing cell viability recovery rates when microalgae cells were cryopreserved during mid-log growth phase (3rd day of cultivation) (Supplementary Table 3).

![Fig. 1. Analysis of cryoprotectants agents efficiency.](image-url)
On the other hand, for *C. sorokiniana*|LBA39 and *C. biconvexa*|LBA40 strains, cells frozen during the 6th day of culture (late-log phase) presented the highest post-thawing viability recovery rates (Supplementary Table 3).

**Fig. 2** shows the response surfaces and contour plots based on the cell viability recovery values obtained for *D. spinosus*|LBA35 strain cryopreserved during mid-log growth phase (3rd day of cultivation) in the presence of different concentrations of the glycerol and PEG 400. Cell viability was significantly affected by glycerol and the PEG 400 concentrations, ranging from 3.9 to 20.75% and from 3.11 to 49.62%, 0 h and 24 h after thawing, respectively. Viability recovery rates increased as the experimental conditions approached the agents’ midpoint (i.e., 5% glycerol + 5% PEG 400) reaching approximately 21% at 0 h and 50% at 24 h after thawing. It is possible to hypothesize that the synergistic effect of glycerol, a cell permeable CPA, and PEG400, a non-permeable CPA, is due to the complementary effect of both agents on the disruption of intracellular ice crystals and promotion of cell dehydration, respectively [7–9].

**Fig. 3** shows the response surfaces and contour plots based on the cell viability recovery values obtained for *C. sorokiniana*|LBA39 strain cryopreserved during late-log phase of growth (6th day of culture) at the different concentrations of glycerol and DMSO. In **Fig. 3A** it is possible to observe that the highest cell viability recovery rates concentrate around the central point. However, a shift towards 0% of glycerol concentration can be observed at cell viability recovery measures 24 h after thawing (**Fig. 3B**). This decrease in cell viability recovery rates in the presence of glycerol 24 h after thawing suggests that this CPA might be toxic to *C. sorokiniana*|LBA39. Therefore, there seems to be an optimum range trend for the cryopreservation of *C. sorokiniana*|LBA39 strain, between 6.7 and 9.7% of DMSO in the absence of glycerol. This result is in agreement with the reported by Morris [38], who observed increased cytotoxicity for *Chlorella* strains as a function of increased glycerol concentrations. Study from Tzovenis et al. [39] also corroborates the efficiency of DMSO as a CPA for the cryopreservation of *Chlorella* species. In addition, the non-synergistic effect of DMSO and glycerol might be related to the fact that both CPAs act intracellularly disrupting ice crystal formation in a similar/redundant mode of action [7,8].

Cryopreservation of *C. biconvexa*|LBA40 was performed using late-log phase cultures (6th day of culture) in the presence of different concentrations of glycerol and PEG 400 (**Fig. 4**). It can be observed that the central conditions of the used cryoprotectants (i.e.: 5% glycerol +5% PEG 400) led to higher cell viability recovery reaching over 70% at 24 h post-thawing (**Fig. 4**).

To our knowledge, this is the first report to describe the use of Central Composite Rotatable Design (CCRD) to study algae cryopreservation. This approach is very useful since it allows empirical testing of interactions among different variables using a reduced set of replicates [26–28]. Nonetheless, there are several reports that describe that the combination of different CPAs promotes greater efficiency in cell recovery compared to the use of each one individually [9,40,41]. Bui et al. [8] evaluated the recovery of 19 strains of microalgae frozen with DMSO or sucrose or the combination of both agents. The effect of sucrose as the sole CPA yielded low cell viability recovery (<10%). DMSO used alone had a significantly higher cryoprotectant effect (68% of the strains were recovered and exhibited viability of ~40%). On the other hand, the combined use of sucrose and DMSO produced an improvement both in terms of the number of strains recovered (100% success) and also in cell viability (~55%). Nakanishi et al. [9], when examining the cryoprotectant efficiency of DMSO, glycerol and ethylene glycol in strains of *C. vulgaris, N. oculata* and *T. tetrathele*, observed little or no cell survival when the agents were used individually. However, the mixture of these CPAs with sorbitol or proline produced beneficial effect for all strains tested. Chong et al. [41] tested either MeOH individually or mixed with sucrose for the cryopreservation of dinoflagellates and found that the combination of both also increased...
Fig. 3. Response surface and contour plots for *C. sorokiniana*|LBA39 strain: After 6 days of cultivation, cells from strain *C. sorokiniana*|LBA39 were frozen in the presence of glycerol (0–10% v/v) and DMSO (0–10% v/v). Cell viability recovery was measured at 0 h (A) and 24 h (B) after thawing.

Fig. 4. Response surface and contour plots for *C. biconvexa* |LBA40 strain: After 6 days of cultivation, cells from strain *C. biconvexa* |LBA40 were frozen in the presence of glycerol (0–10% v/v) and PEG 400 (0–10% v/v). Cell viability recovery was measured at 0 h (A) and 24 h (B) after thawing.
post-thawing cell viability recovery.

3.3. Validation of optimized cryopreservation conditions

The response surfaces and contour plots presented reveal the existence of a region (not a single point) with a high percentage of cell viability recovery after freezing/thawing (Figs. 2 to 4). Therefore, the optimal CPAs combination for cryopreservation of *D. spinosus*|LBA35 and *C. biconvexa*|LBA40 strains can be defined as 5% glycerol +5% PEG 400 (Figs. 2 and 4). While for *C. sorokiniana*|LBA39, the optimal CPA concentration can be defined as 7% of DMSO, using as a criterion a lower concentration of cryoprotective agent within the region of maximum cell viability recovery (6.7% to 9.7% - Fig. 3). In order to validate these definitions, *D. spinosus*|LBA35, *C. biconvexa*|LBA40 and *C. sorokiniana*|LBA39 were cryopreserved using the aforementioned optimal conditions for periods of 15 days and 6 months (Fig. 5). It can be observed that these optimized conditions resulted in statistically significant (p < 0.05) improvements in cell viability recovery for all three strains (Fig. 5). Furthermore, the robust recovery of cell viability observed after 6 months of cryopreservation (Fig. 5C and D) suggests that long term storage using the optimized conditions described in here should be possible. However, it must be highlighted that the temperatures of −80 °C used in this study might reduce viability of materials stored for longer periods of time (i.e.: over 6 months). Therefore, storage in liquid nitrogen (−196 °C) is recommended for long term preservation [9,12,42]. Indeed, successful cryopreservation of algae strains in liquid nitrogen for up to 15 years has been reported [9,42].

In order to verify if the optimized conditions (i.e.: culture age and CPAs type/concentration) obtained for *D. spinosus*|LBA35, *C. biconvexa*|LBA40 and *C. sorokiniana*|LBA39 could be applied to other algae species, subsequent experimental validation was performed upon an additional subset of 12 algal strains (Fig. 6). This subset of algal strains comprises four strains of each of the three morphotypes studied (i.e.: coenobial, coccoid and palmelloid). All 12 strains (100%) could be successfully cryopreserved (Fig. 6) and the post-thawing cell viability recovery rates obtained were similar to those observed in CCRD experiments using *D. spinosus*|LBA35, *C. biconvexa*|LBA40 and *C. sorokiniana*|LBA39 strains (Figs. 2–4). Furthermore, it is possible to observe a correlation between the efficiency of the cryoprotective agents and the algal cell morphology (Fig. 6). Indeed, all the coenobial and palmelloid strains had statistically (p < 0.05) higher rates of cell recovery in the presence of 5% glycerol +5% PEG 400 (Fig. 6A–B and E–F). In addition, 7% of DMSO presented a cryoprotective effect equal to or greater than the 5% glycerol +5% PEG 400 combination for 3 out of 4 coccoid strains analyzed (Fig. 6C–D). Only *Desmodesmus* sp.|LBA36 presented higher cell viability recovery when cryopreserved in the presence of 5% glycerol +5% PEG 400 (Fig. 6C–D). It is important to highlight though that efficient cryopreservation of this strain was also achieved using 7% of DMSO (Fig. 6C–D – Black bars).

The data displayed in Figs. 6 and 7 suggest that both cell morphology and strain phylogenetic origin exert significant influence on the success of cryopreservation. This perception becomes more evident by observing the response to cryopreservation of certain coccoid strains where morphotype and phylogenetic signals seems to “overlap” (i.e.: strains *Desmodesmus* sp.|LBA36, *Scenedesmus ellipticus*|LBA61 and *Scenedesmus ellipticus*|LBA77) (Fig. 7). In such cases, either 7% DMSO or 5% glycerol +5% PEG 400 were capable of providing efficient cryoprotection (Fig. 6). The strains *Desmodesmus* sp.|LBA36, *Scenedesmus ellipticus*|LBA61 and *Scenedesmus ellipticus*|LBA77 exhibit coccoid morphology when cultivated in the laboratory, even though the prototypic morphology of the genera *Desmodesmus* and *Scenedesmus* is a coenobium colony. Recently, several coccoid green microalgae previously assigned to Chlorellales have been reassigned to other taxa, including Sphaeropleales, based on novel evidence from DNA-based identification studies [43–45]. The definition whether morphotype or phylogeny is the main driver in the definition of cryopreservation parameters (i.e.: CPAs type/concentration and culture age), however, remains an issue to be pursued further.

4. Conclusions

The results presented in this study indicate that intrinsic biological factors (i.e.: cell morphology and phylogenetic origin) are determinants of which culture age and cryoprotectant agent type and concentration.
should be used to achieve successful cryopreservation. Indeed, the use of rotational central composite design made possible the optimization of protocols for cryopreserving three major morphotypic groups found among chlorophytes (i.e.: Coenobium, coccoid or palmella forming strains). Although a single universal cryopreservation method for all chlorophytes has not been achieved yet, the information provided here is of valuable practical use since the rapid inspection of algae strain’s predominant morphotype can guide the choice of the most suitable cryopreservation protocol.

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Author contributions

Maiara S. Fernandes; Lorena C. Garcia Calsing; Bruno S. A. F. Brasil contributed to conception, design, analysis and interpretation of the data; collection and assembly of data, article writing, and final approval of the article.

Rodrigo C. Nascimento, Hugo Santana, Priscila B. Morais and Guy de Capdeville contributed to collection and assembly of data and critical revision of the article for important intellectual content.

Maiara Fernandes, Lorena Calsing and Bruno Brasil take responsibility for the integrity of the work as a whole.

Authors agreement

The authors declare their agreement to authorship and submission of the manuscript for peer review.

Conflict of interest

The authors declare that they have no conflict of interest.

No conflicts, informed consent, human or animal rights applicable.
Fig. 7. Relationship among the strain phylogeny, morphology and most efficient cryopreservant agent: The phylogenetic tree was inferred using the General Time Reversible method based on dataset of 135 aligned positions of 15 mtsT2 marker nucleotide sequences. For the analysis, the GTR model with invariable sites (I) and gamma distribution shape parameter (G) (GTR + G + I) was chosen. The bootstrap values (1000 replicates) are shown next to the branches. The strains’ taxonomic assignment, morphology and most efficient CPA are shown.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2018.101402.

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