Chiang Mai J. Sci. 2012; 39(4) : 554-561
http://it.science.cmu.ac.th/ejournal/
Contributed Paper

High Subzero Temperature Preservation of *Spirulina platensis* (*Arthrospira fusiformis*) and Its Ultrastucture

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Received: 2 November 2011
Accepted: 2 July 2012

ABSTRACT

The high subzero preservation of *Spirulina*: *S. platensis* CMU2 and *S. platensis* GD1 was performed by step wise freezing at 25, 4, and -20°C for 30 min. Then they were preserved at -80°C for seven months with four cryoprotectants: dimethyl sulfoxide, horse serum, calf serum and glycerol. The viability of these two strains and cell concentration was not significantly different ($p > 0.05$). The survival of *S. platensis* depended on type and concentration of cryoprotectant and thawing temperature. The best condition for high subzero preservation was obtained by using 5% glycerol as cryoprotectant and rapid thawing at 40°C. After high subzero preservation, their ultrastructure such as cell wall, thylakoid and nucleoplasmic region were changed but cryoprotectant helped to protect these important organelles from freeze-induced damage.

Keywords: dimethyl sulfoxide, calf serum, thylakoid, viability and cooling

1. INTRODUCTION

Spirulina platensis (Arthrospira fusiformis)[1] is a commercially important filamentous cyanobacterium. This alga has been used as a human food supplement and as a feed for fish, poultry, and farm animals [2, 3]. This alga was proved to be a rich source of proteins, minerals, vitamin B12, beta-carotene and essential fatty acids [4].Therefore, it is essential to preserve potentially commercial strains for long term cultivation. Long term preservation protects against genetic selection or drift. It also decreases the probability of contamination of the culture and minimizes errors from handing or labeling mistakes. It is especially useful for strains seldom needed and protects against catastrophic loss of irreplaceable strains [5]. Cryopreservation is ultralow temperature preservation, typically lower than -130°C [6]. This technique has been widely adopted as the preferred in vitro preservation method [7]. Thus, the preservation at the temperature lower than -130°C also required continuously liquid nitrogen supply and an expensive cryogenic storage tank. In addition, the effective protocols

rely on high technology and expensive equipment such as controlled rate freezer or slow programmable freezer [8]. Therefore, our study was interested in the high subzero preservation, which does not required any expensive equipment. Although the high subzero preservation is the freezing at only -80°C, our previous study showed that, the viability of this alga was retained after three months. It was found that these cyanobacteria could survive after being preserved for three month by using DMSO, horse serum and calf serum [9]. However, the three month of high subzero preservation was not long enough for the long term preservation. Thus, the objective of this study was aimed to select the suitable high subzero preservation method to preserve *S. platensis* and observed the viability for longer than three months. In addition, the ultrastructure of the cells after long term high subzero preservation was also studied. The results from this research would provide the procedure for *S. platensis* preservation on the use of simple, cheap and abundant materials with standard laboratory equipment.

2. MATERIALS AND METHODS

2.1 Sample Preparation

Two strains of *Spirulina platensis*; *S. platensis* CMU2 [(Applied Algal Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Figure1A)] and *S. platensis* GD1 [(Green Diamond Company Limited, Chiang Mai, Figure2B)] were cultivated in 600 mL of Zarrouk's medium [10], incubated at 25°C under continuous cool-white fluorescent lamp 8.76 μmole. $m^{-2}.s^{-1}$ with continuous agitation at 120 rpm for 21 days. The growth of these strains was spectrophotometrically measured at 560 nm and cells were counted with a haemacytometer.

2.2 Cryopreservation of *S. platensis*

The study was performed with two initial concentrations of cells: 2.5×10^5 and 1.25×10^5 filaments.mL^{-1} and with four cryoprotectants: dimethylsulfoxide (DMSO), horse serum, calf serum and glycerol used at final concentrations of: 5, 10 and 15% (v/v). A culture of *S. platensis* was mixed with an equal volume of cryoprotectant in a 2 mL cryotube. The tubes were incubated sequentially at 25, 4 and -20°C for 30 min each and maintained at -80°C for subsequent viable monitoring.

2.3 Viability Assays

Viability of samples was checked after 24 hr., 20 and 28 weeks. Samples were thawed using one step protocols in a water bath at 25 or 40°C. The cell sediment was transferred to Zarrouk's medium and incubated for 21 days. Each treatment was performed in 5 duplicates. The viability was measured by counting the number of vials which *Spirulina* could revive after preservation. Percent of viability was calculated from the following equation:

$$\frac{\text{Number of survivable vial in each treatment} \times 100}{\text{Total number of vial in each treatment}}$$

The statistical analysis was performed by Chi-square analysis based on testing data at 95% confidence level. The analysis was performed by SPSS version 14.O for Windows.

2.4 Preparation for the Transmission Electron Microscope (TEM)

The 21-days-old culture of previously frozen *S. platensis* CMU2 was observed for the ultrastructure changes due to cryopreservation. An unfrozen sample was used as control and compared with samples cryopreserved in the presence of 5%

glycerol, or without cryoprotectant. Samples were fixed for 24 hr. at 4°C in 3% glutaraldehyde with 0.1 M phosphate buffer and then washed three times for 10 min in phosphate buffer. Samples were post-fixed in 2% OsO_4 for 1 - 2 hr, and then washed 2 times for 5 min each in 0.1 M phosphate buffer. They were dehydrated twice through 50%, 70% and 85% ethyl alcohol for 5 min each; then 95% alcohol 2 times for 10 min each, and finally 100% alcohol 3 times for 10 min each. Infiltration with resin was by soaking in propylene oxide 2 times for 10 min each, then 1 hr. each in successive solutions containing, respectively, 2:1, 2:2 and 1:2 mixtures of propylene oxide and resin. The sample was then left undisturbed for 24 hr. The samples were embedded in 100% resin at 37°C for 24 hr, then at 45°C for 48 hr. and finally at 60°C for 48 hr. [11]. Thin sectioning was performed using an ultramicrotome. Sections were placed on a copper specimen grid and post-stained with 0.5% uranyl acetate and 0.4% lead citrate [12].

RESULTS AND DISCUSSION

Survival rates of the two strains were very similar in their response to the 5 factors studied, including, initial cell concentration, type and concentration of the cryoprotectants, thawing temperature and preservation time, probably because they were approximately the same size. The length and width of *S. platensis* CMU2 were 461±14.18 μm and 8.50±0.55 μm, respectively while the dimensions of *S. platensis* GD1 were 449± 18.34 μm and 8.25±0.53 μm, respectively (Figures 1A and 1B). Ben-Amotz and Gilboa [5] reported that the viability of marine unicellular algae under cryopreservation did not correlate to the age of culture, but related to the size and specific growth rate.

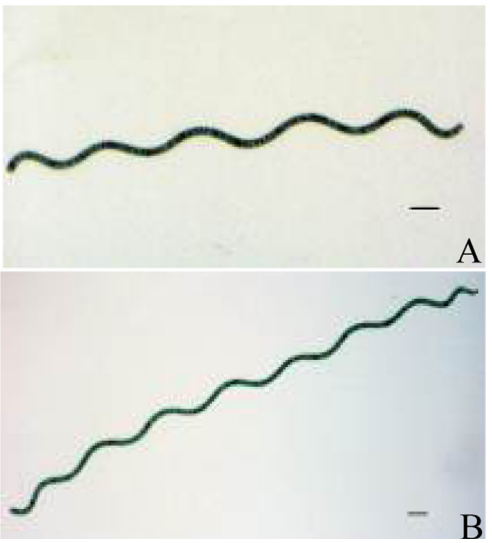

Figure 1. Two strains of *Spirulina platensis* under light microscope 10x *A. S.platensis* CMU2 B. *S.platensis* GD1 Scale bar = 20μm.

The four factors which significantly ($p < 0.05$) affected the viability of *S. platensis* were: type and concentration of cryoprotectant, thawing temperature and cryopreservation period. In comparison with the former study, the viability level at 12 weeks was lower than 24 hr., but higher than 20 and 28 weeks in this study [9]. However, initial cell concentration was not significantly ($p > 0.05$) affected to cyanobacterial survival rate. The higher cell concentration (2.5×10^5 filaments.mL^{-1}) did not show significantly ($p > 0.05$)higher levels of viability than lower cell concentration (1.25×10^5 filaments.mL^{-1}. Lower cell concentrations could lower survival because of toxicity of the cryoprotectant during freezing and thawing. The initial cell concentration of 1.25×10^5 filaments.mL^{-1} was high enough for 20 weeks of preservation. However, the preservation longer than 20 weeks may need the initial cell concentration higher than 2.5×10^5 filaments.mL^{-1}. The result was in accordance with the study by Brand and Diller [13] on *Chlamydomonas reinbardtii,* which was

viable when cryopreserved using cell concentration 2.5×10^{10} cell.mL^{-1}, but did not survive when initial cell concentration was less than 10^4 cell.mL^{-1}.

The presence of a cryoprotectant is important for cell protection during cryopreservation. These cyanobacteria did not survive through freezing and thawing in the absence of a cryoprotectant (control treatment). The concentration of cryoprotectant for preservation of algae and cyanobacteria commonly used is 5 to 10% [14,15]. During the first 24 hr at -80°C 5% DMSO appeared to be the best cryoprotectant, due to its lower toxicity. However, after being frozen for 28 weeks, survival was observed only when 5% glycerol was used as the cryoprotectant (Table 1). This result was similar with the study of Crutchfield *et al.*, who observed that 5% of glycerol was the most effective cryoprotectant followed by 10% dimethysulfoxide and calf serum respectively [16]. This is the first report for successful use of calf serum as cryoprotectant for algal cryopreservation. The result showed that 10% calf serum resulted in similar algal viability levels to DMSO and glycerol after cryopreservation for 20 weeks (Table1).

Table 1. Chi square survival analysis of 2 strains of *S. platensis* after high subzero preservation period from 24 hr. to 28 weeks and its viability.

Factors of viability	number of survival vial/total vial in each treatment (percent of viability)			
	24 hours	20 weeks	28 weeks	Asymptotic significance
1. Strains				0.066
S. platensis CMU2	60/260 (23.1)	11/260 (4.2)	2/260 (0.8)	
S. platensis GD1	79/260 (30.4)	18/260 (7.0)	0/260 (0)	
2. Cell concentration				0.283
2.5×10^5 filament/mL	70/260 (26.9)	20/260 (7.7)	2/260 (0.77)	
1.25×10^5 filament/mL	69/260 (26.5)	9/260 (3.5)	0/260 (0)	
3. Cryoprotectants				0.000*
Control (no cryoprotectant)	0/120 (0)	0/120 (0)	0/120 (0)	
Dimethy sulfoxide				
5%	17/40 (42.5)	3/40 (7.5)	0/40 (0)	
10%	27/40 (67.5)	3/40 (7.5)	0/40 (0)	
15%	14/40 (35.0)	0/40 (0)	0/40 (0)	
House serum				
5%	0/40 (0)	0/40 (0)	0/40 (0)	
10%	0/40 (0)	0/40 (0)	0/40 (0)	
15%	0/40 (0)	0/40 (0)	0/40 (0)	
Calf serum				
5%	2/40 (5.0)	2/40 (5.0)	0/40 (0)	
10%	16/40 (40.0)	5/40 (12.5)	0/40 (0)	
15%	15/40 (35.0)	2/40 (5.0)	0/40 (0)	

Table 1. Continued

Factors of viability	number of survival vial/total vial in each treatment (percent of viability)			
	24 hours	20 weeks	28 weeks	Asymptotic significance
Glycerol				
5%	24/40 (60.0)	10/40 (25.0)	2/40 (5)	
10%	15/40 (37.5)	3/40 (7.5)	0/40 (0)	
15%	9/40 (22.5)	1/40 (2.5)	0/40 (0)	
4. Concentration of cryoprotectants				0.000*
0%	0/160 (0)	0/160 (0)	0/160 (0)	
5%	43/160 (26.9)	15/160 (9.4)	2/160 (1.3)	
10%	58/160 (36.3)	10/160 (6.3)	0/160 (0)	
15%	38/160 (23.8)	3/160 (1.9)	0/160 (0)	
5. Temperature of thawing				0.047*
25°C	60/260 (23.1)	15/260 (5.8)	0/260 (0)	
40°C	79/260 (30.4)	14/260 (5.4)	2/260 (0.8)	
6. Preservation period				0.000*
	139/520 (26.7)	29/520 (5.6)	2/520 (0.4)	

*Significant difference $p < 0.05$

Thawing by putting the cultures at 25°C and 40°C indicated that thawing at 40°C resulted in significantly higher viability, as has been previously reported [17,18,19]. Rapid thawing has the advantage of minimizing exposure time of cryoprotectant and reduction of cells damage by ice.

The success of a cryopreservation procedure requires slow freezing. *Isochrysis galbana* was cryopreserved at -20°C and showed 7.3% viability while cryopreserved at -80°C did not show viability [18]. This study also gave a success of cryopreservation by using step wise temperature decrease. The method was effective, cheap and did not require high technology or expensive machine.

Ultrastructure was studied in *S. platensis* CMU2 because the viability of two *Spirulina* strains was not significantly different. The cell ultrastructure changed after freezing and thawing (Figure 2b, 2c) when compared to the unfrozen control (Figure 2a). The ultrastructure of non-cryoprotective cells were destroyed by intracellular ice-crystal growth [6] and viability levels were diminished. The cell wall was changed and the damage to outer cell wall surface was observed after freezing and thawing without any cryoprotectant. However, with glycerol as cryoprotectant, the cell wall was protected (Figure 2b).

The Thylakoid membranes of *S. platensis* were also protected by glycerol. Normally, the thylakoid contains two regularly unit membranes [13]. The outer membrane contains proteins and the inner layer contains pigment associated with photosynthesis and enzymes involved [20].

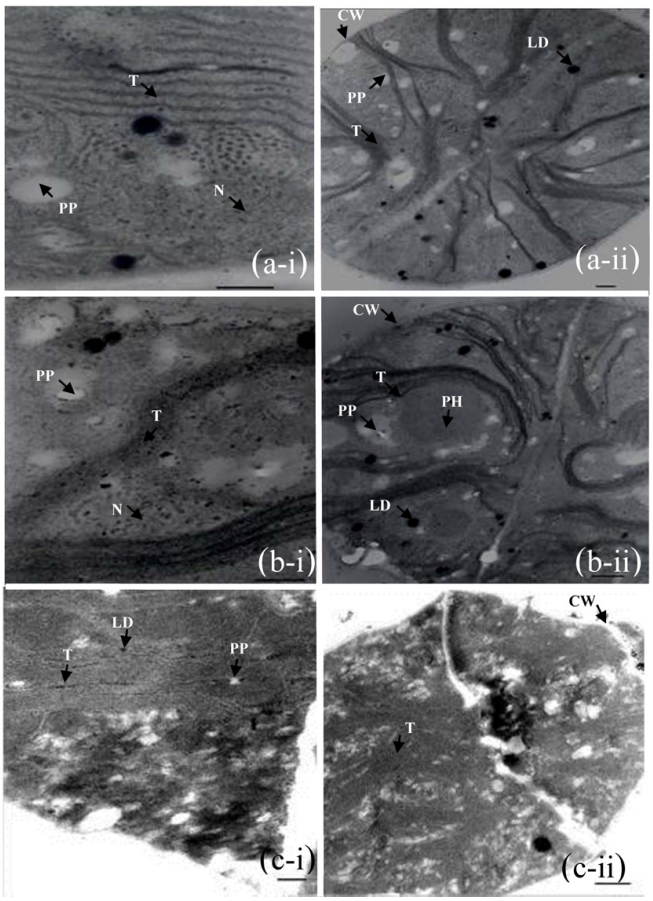

Figure 2. Ultrastructure of *S. platensis* CMU2:**(a-i)** longitudinal section of *S. platensis* CMU2 unfrozen **(a-ii)** cross section of *S. platensis* CMU2 unfrozen **(b-i)** long section of *S. platensis* high subzero preserved for 24 hr. with 5% glycerol **(b-ii)** cross section of *S. platensis* high subzero preserved for 24 hr. with 5% glycerol **(c-i)** long section of *S. platensis* CMU2 high subzero preserved without cryoprotectant for 24 hr. **(c-ii)** cross section of *S. platensis* CMU2 high subzero preserved without cryoprotectant for 24 hr. Scale bar = 5 μm.
CW=cell wall, T= thylakoild, N= nucleoplasmic region with DNA and ribosome, PP= polyphosphate granule, PH= polyhedral bodies and LD=Lipid droplet.

The membrane also changed after cryopreservation without cryoprotectant (Figure 2c) affecting the breakdown and narrow thylakoid spacer, which might result in less photosynthesis and lower the viability of these cells.

Other changes occurred in the nucleoplasmic region and in ribosome structure [20, 21]. Endoplasmic Reticulum and ribosome disappeared after cryopreservation in the presence of glycerol (Figure 2: b-i, b-ii), but without cryoprotectant (Figure 2: c-i, c-ii), these organelles are distributed throughout the cell. Another apparent change in the cell preserved in 5% glycerol was the presence of dispersed polyphosphate granules (PP).

Two structures in the cytoplasm which accumulated/store excess nutrients that were unchanged after cryopreservation

were lipid droplets (LD) and polyhedral bodies (PH). Excess nutrients are stored as insoluble polymers localized in inclusion bodies. The accumulation of excess nutrients may be of critical importance in sustaining the cyanobacteria in a variety of freshwater habitats when the exogenous sources of nutrients such as nitrogen and phosphorus, are limited [21].

CONCLUSION

The viability of the two *S. platensis* strains following freezing and thawing was not significantly ($p > 0.05$) different. However, viability was reduced significantly during prolonged storage at -80°C. Simple and readily available laboratory equipment in laboratory could preserve *S. platensis* for up to 7 months. This is the first study in using calf serum as cryoprotectant and it could preserve *S. platensis* with viabilities as high as with DMSO and glycerol. High subzero preservation without cryoprotectant significantly damaged internal structures, lysed cell wall, distorted the space between thylakoid membranes, diffused polyphosphate granules and destroyed the nucleoplasmic region. From the results, simple and available equipment in the laboratory could be practical cryopreserving other kinds of algae and cyanobacteria.

ACKNOWLEDGEMENTS

The authors would like to thank the Conservation and Utilization of Biodiversity Project and Graduated School, Chiang Mai University for providing a research grant.

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