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A CRYOPRESERVATION PROTOCOL FOR THE MALAYSIAN FRE WATER MICROALGAL STRAIN

Nor Afiqah, A.[1†] **--- Siti Saizah, M.S.**[2] **--- Ernie Eileen, R.R.**[3]
[1,2,3]*Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Shah Alam, Malaysia*

ABSTRACT

Microalgae is important in many biotechnological exploitations in producing valuable products, services and processes. The technology of cryopreservation is useful in the long-term storage of many microalgal strains able to survive post cryopreservation. In this study, Chlorella vulgaris was utilised to determine the effects of different concentrations of a cryoprotectant to preserve the fresh water microalgal strain employing three types of protocol. Dimethyl sulfoxide (DMSO) at different concentrations of 0%, 10%, 20% and 30% were added to the microalgal suspension in three types of protocol. Protocol 1 involved direct plunging into liquid nitrogen, Protocol 2 is slow cooling to -80°C and Protocol 3 is slow cooling to -20°C. The absorbance value at 540 nm was used as a measurement to determine the growth post cryopreservation. The absorbance value recorded with 30% DMSO in Protocol 2 was the highest at 0.387 $\pm$ 0.015 and was significant at $p<0.05$ compared to Protocol 1 and 3. The best protocol for post cryopreservation growth of Chlorella vulgaris was Protocol 2 using slow cooling to -80 °C treated with 30% DMSO as the cryoprotective agent.

Keywords: Absorbance value, Cryopreservation, Cryoprotectant, Dimethyl sulfoxide (DMSO), Microalgae, Protocol

Contribution/ Originality

The paper's primary contribution is finding that the use of 30% DMSO as a cryoprotectant produced the optimal results for the viability of the microalgae. This study documents that the microalgae cryopreserved using Protocol 2, which involved slow cooling in the -80°C freezer showed the best protocol in the cryopreservation of the fresh water microalgal strain.

1. INTRODUCTION

Cryopreservation is a process whereby the cells or the whole tissue are preserved by cooling to an ultralow temperature of -196 °C such that it remains capable of survival upon thawing (Roshani *et al.*, 2011). In addition, the techniques of cryopreservation in liquid nitrogen is beneficial because of its wide range, long-term storage and little mutation on all the cells (Xue-

† Corresponding author

Ling *et al*., 2008). Moreover, cryopreservation is a practical method for maintaining a broad range of microalgae over a long period of time (Gwo *et al*., 2005). Using the cryoprotectants, or chemicals that protect the cells during the freezing, it will minimize the effects of ice crystal formation and increased solute formation. This research is beneficial in optimising a successful protocol for the cryopreservation of the Malaysian fresh water microalgal strain. The objectives of the study were to determine the effects of different concentrations of a cryoprotectant to cryopreserve fresh water microalgal strain and to compare three different protocols for the cryopreservation of fresh water microalgal strain.

2. MATERIALS AND METHODS

2.1. Materials

The stock culture of *Chlorella vulgaris* were obtained from the Environment Control Laboratory, School of Chemical Engineering, Universiti Sains Malaysia, Pulau Pinang.

2.2. Growth Medium

Chlorella vulgaris were grown in Bold's Basal Medium(BBM) comprising of fourteen different chemicals at the required molarity (Connon, 2007). The microalgal suspension was placed on an orbital shaker at 160 rpm at room temperature under continuos ilumination of light pendarfluor lamps. The microalgal suspension was used for the experiment at the log phase as the cells were actively growing.

2.3. Cryoprotectant Solution

Dimethyl sulfoxide (DMSO) was used in this experiment as a cryoprotectant with different concentrations of 0%, 10%, 20% and 30%. DMSO was filter sterilized using a membrane filter. The cryoprotectant was diluted with the BBM before being added to the microalgal suspension. This will minimized the potentially deleterious effects of chemical reactions and to ensure a uniform exposure of the cryoprotectant during the addition to the microalgal suspension (Simione, 2009). Moreover, it will also reduce the potential of toxic effects to the cells.

2.4. Cryopreservation Protocol

Three freezing protocols were tested in this research on the Malaysian fresh water microalgal strain. Protocol 1 involved a rapid cooling technique whereby the microalgal suspension in the cryovials was directly plunged into liquid nitrogen at -196°C. Protocol 2 is a two-step slow cooling process, whereby cryovials containing algal samples were placed in the Nalgene™ Mr Frosty freezing container (at room temperature), filled with isopropyl alcohol. This device allowed a cooling rate of -1°C min^{-1}. The Mr Frosty container were then placed into a -80°C freezer for at least 1.5 hours before being transferred to the liquid nitrogen storage tank.

Protocol 3 involved cryovials being placed into a Nalgene™ Mr Frosty freezing container (at room temperature), filled with isopropyl alcohol which were then placed into a -20°C freezer. The samples were placed in the freezer for at least 24 hours before being transferred to the liquid nitrogen storage tank. The samples of *Chlorella vulgaris* were left in liquid nitrogen for at least one week prior to thawing.

2.5. Thawing Process

After storage periods of one week, the cryovials were taken out from the liquid nitrogen tank and transferred to the water bath at 35°C for 2 minutes. The mixtures were agitated to ensure that it will be completely thawed. To minimize the risk of contamination during reconstitution, the external surface of the cryovials were disinfected by using 70% ethanol prior to opening. The cell suspension that was completely thawed were centrifuged for 10 minutes at 10 000 rpm. The supernatant was removed and the cells resuspended with the BBM to remove any residual cryoprotective chemicals. The microalgal supension was centrifuged again to ensure that all the cryoprotectant was completely removed to avoid toxicity to the microalgal suspension.

2.6. Evaluation of the Survival Post Cryopreservation

The absorbance value at 540 nm was used as a measurement to determine the growth post cryopreservation. By using the spectrophotometer at wavelength of 540 nm, the absorbance value of the chlorophyll content in the microalgal suspension was determined. The values obtained indicate the survival and growth of the cell post cryopreservation.

3. STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS Software version 20, (2011). The absorbance value at different concentrations for different protocols (Mean±SEM) were calculated using one-way ANOVA. The optimal concentration of DMSO was evaluated, and the best protocol for the viability of algal cells post cryopreservation was determined.

4. RESULTS AND DISCUSSION

The absorbance value with 30% DMSO in Protocol 2 showed the highest reading at 0.387 ± 0.015 and was significant at $p<0.05$ compared to Protocol 1 and 3 (Table 1).

Tukey post-hoc test: [a] significant ($p<0.05$) compared with Protocol 2 and Protocol, [b] significant ($p<0.05$) compared with Protocol 1 and Protocol 3 and [c] significant ($p<0.05$) compared with Protocol 1 and Protocol 2

Table-1. The absorbance value (540 nm) of different concentrations of DMSO with different protocols (Mean ± SEM)

Concentration of DMSO	Protocol 1	Protocol 2	Protocol 3
0%	0.047 ± 0.005^{bc}	0.227 ± 0.003^{ac}	0.098 ± 0.003^{ab}
10%	0.134 ± 0.002^{bc}	0.241 ± 0.015^{ac}	0.206 ± 0.004^{ab}
20%	0.178 ± 0.011^{bc}	0.259 ± 0.009^{a}	0.222 ± 0.009^{a}
30%	0.242 ± 0.006^{b}	0.387 ± 0.015^{ac}	0.251 ± 0.008^{b}

Moreover, the slow cooling effect to -80°C done in Protocol 2 showed the highest absorbance value (Figure 1). The findings of this study is in accordance with that of slow cooling to -80°C for the viability of cells post cryopreservation (Roshani *et al.*, 2011). The two-step method is the conventional method usually used to preserve microalgae and cyanobacteria (Day and Brand, 2005). The damage to cells was prevented because the reduced cooling rate allowed sufficient time for osmotic equilbrium to be maintained. It also mimimized the formation of lethal intracelular ice which took place in rapid cooling in Protocol 1. When a cell is cooled rapidly, it may cause thermal shock (Brockbank *et al.*, 2004) as observed in Protocol 1 which involved direct plunging into liquid nitrogen.

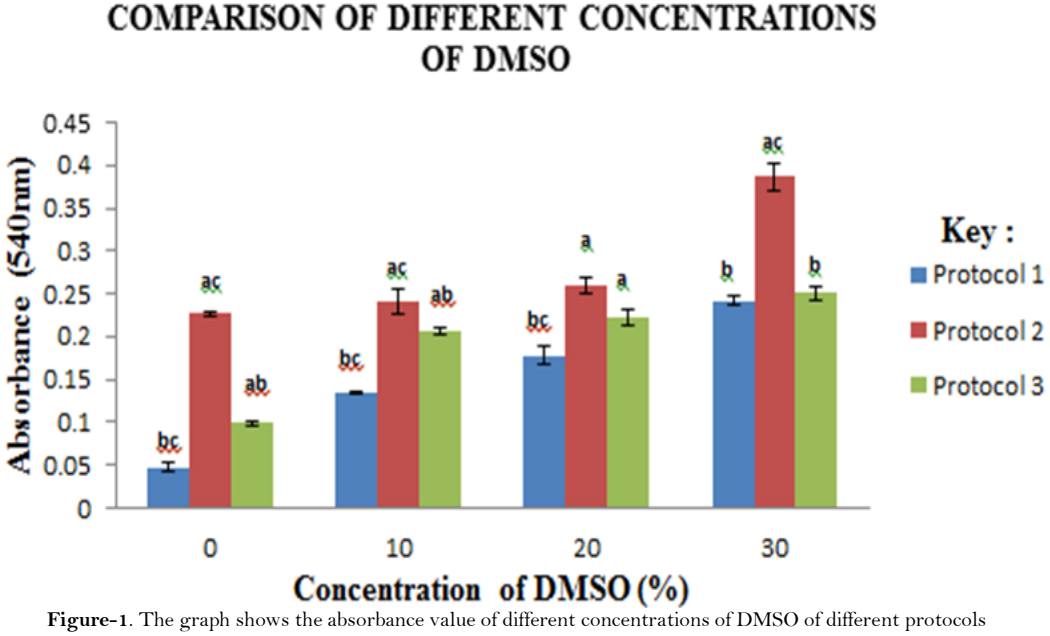

Figure-1. The graph shows the absorbance value of different concentrations of DMSO of different protocols

Cryoprotectants were added to prevent cell damage and the cryoprotectant which has a low molecular weight such as methanol and DMSO were able to penetrate the plasma membrane and equilibrated the solute concentration inside and outside the cell (Acker *et al.*, 2004). Thus, it was important in this study to ensure that the cell survived post cryopreservation by using this type of cryoprotectant. There were no surviving cells observed by using 2.5% or 5% concentration of

DMSO alone (Nakanishi *et al*., 2012). In this study, different concentrations were used and results indicated that the highest concentration of 30% DMSO produced the optimal result (Figure 2). As the microalgal cell suspension start to freeze, the formation of ice crystal developed. The plasma membrane may be punctured due to the formation of ice crystals and may lead to cell death (Hubalek, 2003). However, the presence of DMSO at the optimal concentration protected the cells by penetrating the membrane and caused the cells to be less prone to being punctured. Besides, it also interrupted the lattice of ice such that only a few crystals were formed.

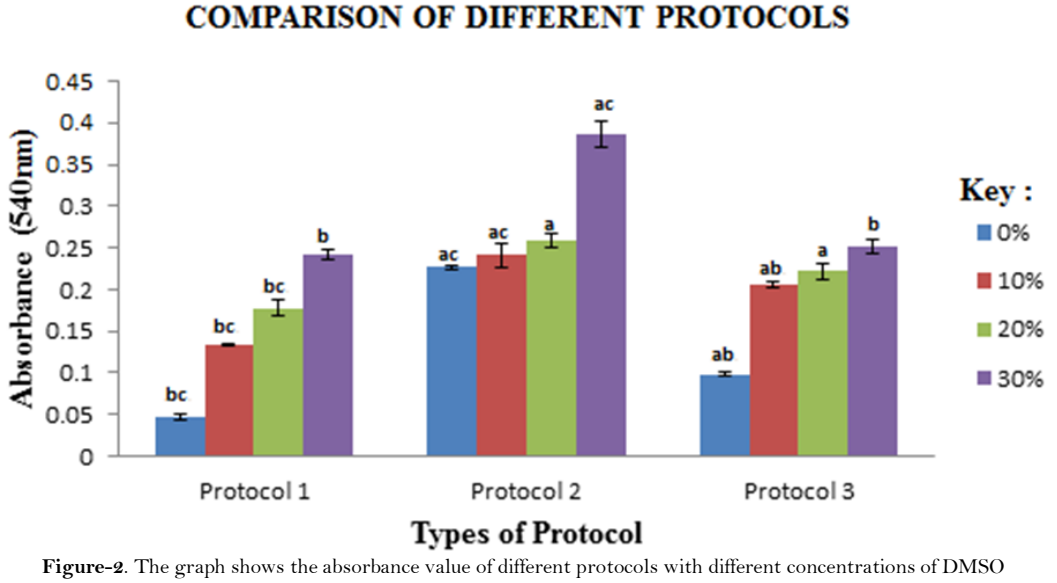

Figure-2. The graph shows the absorbance value of different protocols with different concentrations of DMSO

5. CONCLUSION

The study showed that 30% of DMSO as a cryoprotective agent produced the optimal result for the three types of protocol tested. The best protocol for the cryopreservation of *Chlorella vulgaris* is Protocol 2 using slow cooling to -80 °C as results indicated a high absorbance value.

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