



Optimization of cryopreservation method for toxin-producing cyanobacteria

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ABSTRACT

Aims: The process of isolating cyanobacteria from various environments and ecosystems can be challenging, consuming a lot of time and laborious. Therefore, to maintain cyanobacterial culture, including toxin-producing cyanobacteria, for a longer period, the best preservation method should be developed and established. This study was conducted to assess the effectiveness of cryopreservation of isolated toxin-producing cyanobacterial strains from aquatic environments using deep freezing with methanol as the cryoprotectant.

Methodology and results: Twelve strains of cyanobacteria were isolated from various locations in Malaysia and inoculated in BG11 media supplemented with 0%, 5%, and 10% methanol and kept in -20 °C freezer for one, two and three months. The strains' viability was observed for one month at room temperature after being preserved in the freezer. The evaluated cyanobacteria exhibited different responses to the cryopreservation protocols following their classification and group. Storage in the -20 °C freezer was not suitable for the filamentous cyanobacterial strain, *Leptolyngbya frigida* ANT.L52B.3, while five out of the twelve strains tested were still viable only when cryopreserved in the presence of methanol. Meanwhile, a total of five strains (*Synechococcus* sp. EO68, *Synechococcus* sp. M1, *Nodosilinea* cf. *nodulosa* LEGE 10377, *Cephalothrix komarekiana* SAG 75.79 and *Oscillatoria* sp. OF9) responded well with methanol, showing high post-thaw viability even after three months of preservation.

Conclusion, significance and impact of study: Our results demonstrated that methanol is suitable for preserving most toxin-producing cyanobacterial strains tested in this study, offering a practical alternative to costly and time-consuming maintenance processes while conserving valuable genetic resources.

Keywords: Cryopreservation, effectiveness, methanol, toxin-producing cyanobacteria, viability

INTRODUCTION

Isolation and purification processes for cyanobacteria are time-consuming, labour-intensive, and costly (Phukan *et al.*, 2015). Maintaining pure and axenic cultures through effective preservation techniques is vital for research and biotechnological applications, as some cyanobacteria produce toxins that impact the ecosystems (Ribeiro *et al.*, 2020), while a number of them serve as promising resources for renewable energy (Singh *et al.*, 2023) and natural products (Baunach *et al.*, 2024). Thus, preservation methods must ensure cell viability and maintain physiological, morphological, and genetic characteristics during the storage periods (Ali *et al.*, 2021). Traditionally, cyanobacterial cultures have been

maintained by serial subculturing in agar or liquid media. However, this approach is not only time-consuming, laborious, and high cost, but also increases the risk of species contamination and genetic drift due to repeated subculturing (Iwamoto *et al.*, 2012). Besides serial transfer, slant agar has also been used regularly for subculturing, but this technique has similar drawbacks as serial transfer (Redkina and Temraleeva, 2025).

Presently, cryopreservation is the most common technique in the preservation of cyanobacteria, conserving their biological and genetic properties over a longer period of time (Day, 2007; Esteves-Ferreira *et al.*, 2013). The general principle underlying the cryopreservation technique is freezing and storage of cells at very low temperature (Bojic *et al.*, 2021) and has

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proven to result in a high success rate for various groups of cyanobacteria (Gaget *et al.*, 2017). The benefits of cryopreservation include the conservation of the wild properties of strains, retention of cell viability, and preservation of the morphological and biochemical characteristics of immobilised cells for a longer period (Phukan *et al.*, 2015). Cryopreservation also minimizes the usage of chemicals and manpower, reduces potential risks of contamination with other microorganisms, and saves glassware and storage space. In addition, it retains genetic and phenotypic stability by keeping cells in a near-dormant state (Syiem and Bhattacharjee, 2010). Hence, strains or species loss can be minimized owing to low contamination and genetic drift (Esteves-Ferreira *et al.*, 2013). Deep freezing with intracellular cryoprotectants is a common method used in cryopreservation. Cryoprotectant is referred to as a chemical used in the cell suspension for an extended period to protect cells from damage when frozen (Tutrina and Zhurilov, 2024).

Many cryoprotectants have been used or tested, including dimethyl sulphide (DMSO), methanol (Redkina and Temraleeva, 2025), sucrose, and large polymer molecules (Oluwatosin *et al.*, 2022) and glycerol (Senaratne and Jayaweera, 2024). Among those mentioned, dimethyl sulphide (DMSO), methanol, and glycerol are the most effective (Park, 2006; Guo *et al.*, 2020). Both methanol and DMSO inhibit ice formation when cells freeze by penetrating the cells. Methanol and DMSO freely permeate cell membranes due to their low hydrophilicity and molecular weight (Youn and Hur, 2009). These properties allow them to interrupt ice crystal formation by forming hydrogen bonds with water molecules (Bui *et al.*, 2013). Such permeable cryoprotectants recover the cell suspension in cryopreservation by minimizing the changes in cell volume and damage from intracellular ice formation and preventing cell breakage (Núñez-Vázquez *et al.*, 2011). Therefore, this study aims to evaluate the effectiveness of methanol as a cryoprotectant for the long-term preservation of toxin-producing cyanobacteria.

MATERIALS AND METHODS

Cyanobacterial strains

In this study, twelve toxin-producing cyanobacterial strains displaying diverse morphological characteristics and belonged to four taxonomic orders, namely Nostocales, Synechococcales and Oscillatoriales (Komárek *et al.*, 2014) were evaluated. The cyanobacteria used exhibited various cell morphologies, including unicellular, filamentous, and heterocystous forms. The detailed description of the strains and their characteristics are tabulated in Table 1.

Table 1: Cyanobacterial strains used in this study and their characteristics.

Orders	Cyanobacterial names	Morphologies	
Synechococcales	<i>Synechococcus</i> sp. EO68	Unicellular	
	<i>Synechococcus</i> sp. M1	Unicellular	
	<i>Synechocystis</i> sp. CCALA 700	Unicellular	
	<i>Leptolyngbya frigida</i> ANT.L52B.3	Filaments non-heterocystous	
	<i>Leptolyngbya</i> sp. D1C10	Filaments non-heterocystous	
	<i>Nodosilinea</i> cf. <i>nodulosa</i> LEGE 10377	Filaments non-heterocystous	
	<i>Limnothrix redekei</i> BTA657	Filaments non-heterocystous	
	<i>Limnothrix</i> sp. B15	Filaments non-heterocystous	
	Oscillatoriales	<i>Cephalothrix komarekiana</i> SAG 75.79	Filaments non-heterocystous
		<i>Oscillatoria</i> sp. OF9	Filaments non-heterocystous
<i>Aerosakkonema funiforme</i> strain Lao26		Filaments non-heterocystous	
Nostocales	<i>Scytonema</i> sp. U-3-3	Filaments heterocystous with false branching	

Sample preparation

The tested cyanobacteria were cultured in 250 mL conical flasks containing 100 mL BG11 media at ambient temperature with 24 h light of 30 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ supplied by fluorescent lamps. One mL of the cyanobacterial culture at the late logarithmic stage of growth was aseptically pipetted into sterile 1.5 mL microcentrifuge tubes, with three replicates per strain. Then the samples were spun down for pellet collection, and the supernatant was completely removed. To assess how cryoprotectants affect cyanobacteria, samples were subjected to two treatments, with or without cryoprotectant.

For treatment with cryoprotectant, methanol was added to autoclaved liquid BG11 media at two different concentrations, 5% and 10%, while for treatment without cryoprotectant, methanol was omitted. One mL of each prepared solution was aliquoted into microcentrifuge tubes containing the cell pellets, with triplicates for each treatment. The tubes were then capped, sealed, and stored in the -20 °C mechanical freezer for the period of either one, two or three months until the recovery test was performed. For control, 1 mL of untreated cell culture was

transferred into BG11 medium to validate the viability of pre-culture.

Thawing procedure

The thawing process used in this study is according to Brand (2003). Cryopreserved samples were retrieved from the -20 °C freezer at the end of each cryopreservation period and rapidly cooled down at room temperature before being cultured in new culture media.

To assess the viability of the cyanobacteria, samples were thawed at room temperature until the ice had completely melted. The cyanobacterial cells were then gently centrifuged at the lowest possible speed to minimize damage. The supernatant was discarded, and 1 mL of BG11 broth medium was added to resuspend the cells. The tube caps were slightly loosened for gas exchange to happen, and the samples were incubated in the dark for 24 h. Following incubation, the tubes were centrifuged again, supernatant discarded, and the pellets were pipetted into 50 mL of universal bottles containing 20 mL of BG11 media. All procedures were repeated three times.

Recovery test and post-thaw viability analysis

The cell suspensions in fresh medium were incubated under the same conditions as the pre-culture. The growth of the revived strain was monitored for one month by measuring the chlorophyll *a* content. Chlorophyll *a* content was quantified according to Ritchie (2006).

An aliquot of culture (e.g., 1.5 mL) was transferred into a microcentrifuge tube and centrifuged at high speed (approximately 10,000-12,000 × g) for several minutes to pellet the cells. The supernatant was carefully removed, and the centrifugation step was repeated if necessary to ensure complete removal of the medium. The resulting pellet was resuspended in an equal volume of 90% methanol containing magnesium carbonate (10 mg/L) and incubated in the dark at room temperature for one hour to extract chlorophyll *a*. The extract was then centrifuged again to remove cell debris, and the absorbance of the supernatant was measured at 665 nm. The extraction solvent was used as the blank.

$$\text{Chlorophyll } a \text{ (mg/L)} = A_{665} \times 12.9447$$

where A_{665} is the absorbance at 665 nm, and 12.9447 is the specific absorption coefficient.

Viability of the cell was assessed based on the ability of the cryopreserved samples to completely revive and demonstrate full growth compared to the control culture after inoculation.

RESULTS AND DISCUSSION

Cyanobacterial strains exhibit varying responses to cryopreservation methods. While some methods are broadly applicable to a wide class of cyanobacterial

strains, certain strains behave differently (Day, 2007). Thus, there is a need to establish a standardized cryopreservation protocol and to understand the underlying mechanisms used by cyanobacterial strains to withstand distinct and different forms of dehydration.

The viability of unicellular cyanobacterial strains following freezing at -20 °C is shown in Table 2. It was observed that all tested unicellular cyanobacterial strains showed post-thaw viability even after three months of storage. A lag phase was observed in all cryopreserved samples, regardless of whether a cryoprotectant was used. Strains that were not cryopreserved resumed growth without a lag phase, which is similar to the control. Samples preserved without methanol showed a longer lag phase compared to those preserved with cryoprotectant (Figure 1). Some unicellular cyanobacteria showed a prolonged lag phase with longer cryopreserved times. All strains cryopreserved with methanol successfully revived after one, two, and three months of preservation at -20 °C (Table 2). Among the tested unicellular strains, *Synechococcus* sp. E068 and *Synechocystis* sp. CCALA 700 failed to revive without methanol, while another strain (*Synechococcus* sp. M1) was revived even in the absence of cryoprotectant. Interestingly, *Synechococcus* sp. M1 showed better growth when cryopreserved without methanol after two and three months of storage. Meanwhile, for cryopreservation with methanol, a delay in the growth was observed as the cryopreservation period increased.

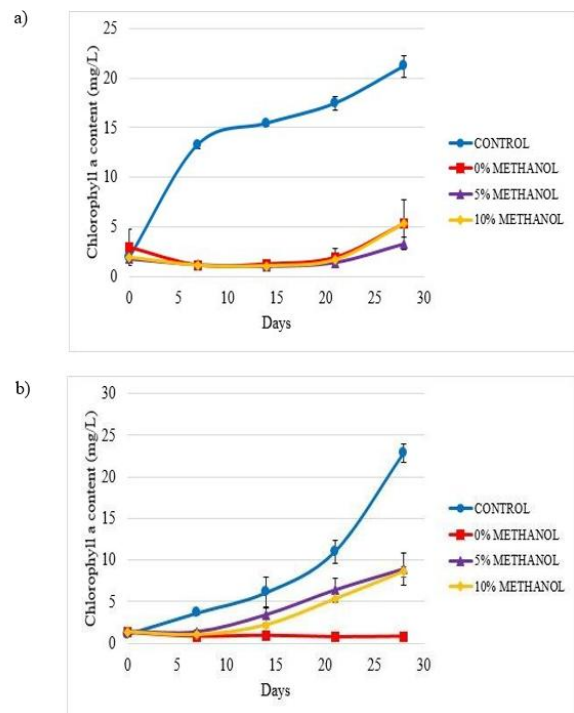


Figure 1: Growth of unicellular cyanobacterial species: a) *Synechococcus* sp. M1, b) *Synechocystis* sp. CCALA 700 after 3 months of cryopreservation.

Table 2: The post-thaw viability of unicellular cyanobacterial species according to the preservation time.

Strains	Concentration of methanol (%)	Viability		
		1 month	2 months	3 months
<i>Synechococcus</i> sp. EO68	C (0)	-	-	-
	C (5)	+	+	+
	C (10)	+	+	+
<i>Synechococcus</i> sp. M1	C (0)	+	+	+
	C (5)	+	+	+
	C (10)	+	+	+
<i>Synechocystis</i> sp. CCALA 700	C (0)	-	-	-
	C (5)	+	+	+
	C (10)	+	+	-

C (0) cryopreservation without methanol, C (5): cryopreservation with 5% methanol and C (10): cryopreservation with 10% methanol, +: full growth after inoculation as observed in the control culture, -: no sign of growth for a month.

A lag phase was observed in all revived non-heterocystous filamentous strains. In contrast, strains that were not preserved in -20 °C resumed growth immediately without a lag phase, as was observed in the control strains. Samples cryopreserved without cryoprotectant showed a longer lag phase compared to those cryopreserved with methanol. When longer cryopreservation periods were applied, and a prolonged lag phase was observed in some strains (Figure 2). The viability of eight different non-heterocystous cyanobacterial strains after freezing in -20 °C is summarized in Table 3. Among the tested strains, *L. frigida* ANT.L52B.3 did not recover under any condition. *Nodosilinea* cf. *nodulosa* LEGE 10377 is the only strain that remained viable after three months of storage regardless of absence or presence of cryoprotectant. This indicates that this strain does not require cryopreservative agents for preservation at low temperatures. Conversely, *Leptolyngbya* sp. D1C10 and *A. funiforme* strain Lao26 revived only when methanol was used.

Strains that remained viable after three months of cryopreservation are *Leptolyngbya* sp. D1C10, *Nodosilinea* cf. *nodulosa* LEGE 10377, *C. komarekiana* SAG 75.79, and *Oscillatoria* sp. OF9. Notably, *C. komarekiana* SAG 5.79 and *Oscillatoria* sp. OF9 revived only with methanol for three months of storage, highlighting the role of methanol as an effective cryoprotectant. For *Leptolyngbya* sp. D1C10 revival occurred only with 5% methanol, but not without methanol or 10% methanol after two and three months of preservation. These results suggest that optimal methanol concentration is critical for this strain. Lack of methanol may lead to cell rupture, while excessive methanol (10%) can be toxic. Meanwhile, *L. redekei* BTA657, *Limnothrix*

sp. B15 and *A. funiforme* strain Lao26 are the strains that showed no viability after three months under any condition, indicating that longer cryopreservation period is unsuitable for these strains.

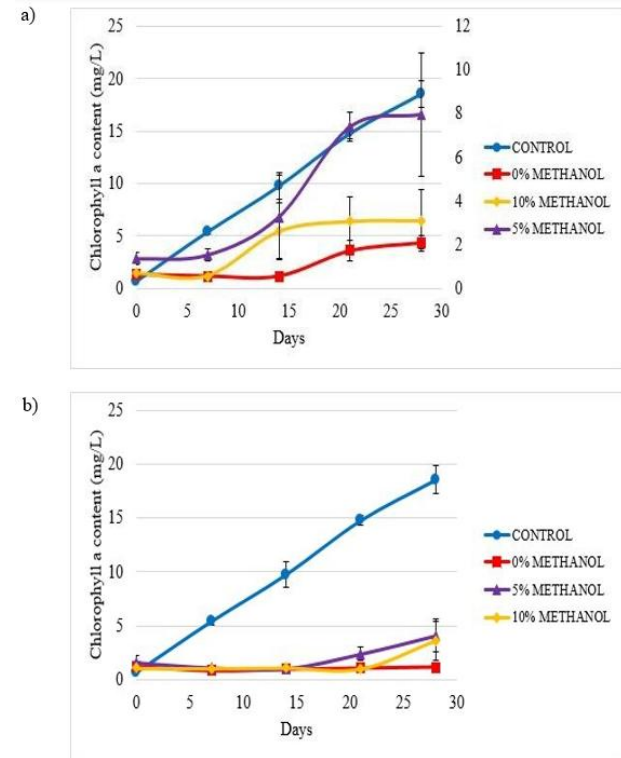


Figure 2: Growth of filamentous cyanobacterial species: a) *Oscillatoria* sp. OF9 after 2 months of cryopreservation, b) *Oscillatoria* sp. OF9 after 3 months of cryopreservation.

Table 3: The post-thaw viability of filamentous cyanobacterial species according to preservation time.

Strains	Concentration of methanol (%)	Viability		
		1 month	2 months	3 months
<i>Leptolyngbya frigida</i> ANT.L52B.3	C (0)	-	-	-
	C (5)	-	-	-
	C (10)	-	-	-
<i>Leptolyngbya</i> sp. D1C10	C (0)	-	-	-
	C (5)	+	+	+
	C (10)	+	-	-
<i>Nodosilinea</i> cf. <i>nodulosa</i> LEGE 10377	C (0)	+	+	+
	C (5)	+	+	+
	C (10)	+	+	+
<i>Limnothrix redekei</i> BTA657	C (0)	+	-	-
	C (5)	+	+	-
	C (10)	+	+	-

Continued Table 3.

<i>Limnothrix</i> sp. B15	C (0)	+	-	-
	C (5)	+	-	-
	C (10)	+	-	-
<i>Cephalothrix komarekiana</i> SAG 75.79	C (0)	+	-	-
	C (5)	+	+	+
	C (10)	+	+	+
<i>Oscillatoria</i> sp. OF9	C (0)	+	+	-
	C (5)	+	+	+
	C (10)	+	+	+
<i>Aerosakkonema funiforme</i> strain Lao26	C (0)	-	-	-
	C (5)	+	-	-
	C (10)	+	-	-

C (0) cryopreservation without methanol, C (5): cryopreservation with 5% methanol and C (10): cryopreservation with 10% methanol, +: full growth after inoculation as observed in the control culture, -: no sign of growth for a month.

Table 4 shows the effects of cryopreservation methods on a selected heterocystous filamentous cyanobacterial strain. After one month of storage, *Scytonema* sp. U-3-3 revived only when methanol was used as a cryoprotectant but exhibited slower growth at both 5% and 10% methanol. A similar trend was observed after two months of storage, with revival occurring only in the presence of methanol. However, *Scytonema* sp. U-3-3 showed no viability after three months of cryopreservation under any condition, indicating no significant advantage of using methanol for a longer period of storage. The lag phase was also observed in the revived strain, whereas the control strain that was not preserved in -20 °C resumed growth without a lag phase. Longer cryopreservation periods were associated with an extended lag phase (Figure 3).

Table 4: The post-thaw viability of heterocystous cyanobacterial species according to the preservation time.

Strains	Concentration of methanol (%)	Viability		
		1 month	2 months	3 months
<i>Scytonema</i> sp. U-3-3	C (0)	-	-	-
	C (5)	+	+	-
	C (10)	+	+	-

C (0) cryopreservation without methanol, C (5): cryopreservation with 5% methanol and C (10): cryopreservation with 10% methanol, +: full growth after inoculation as observed in the control culture, -: no sign of growth for a month.

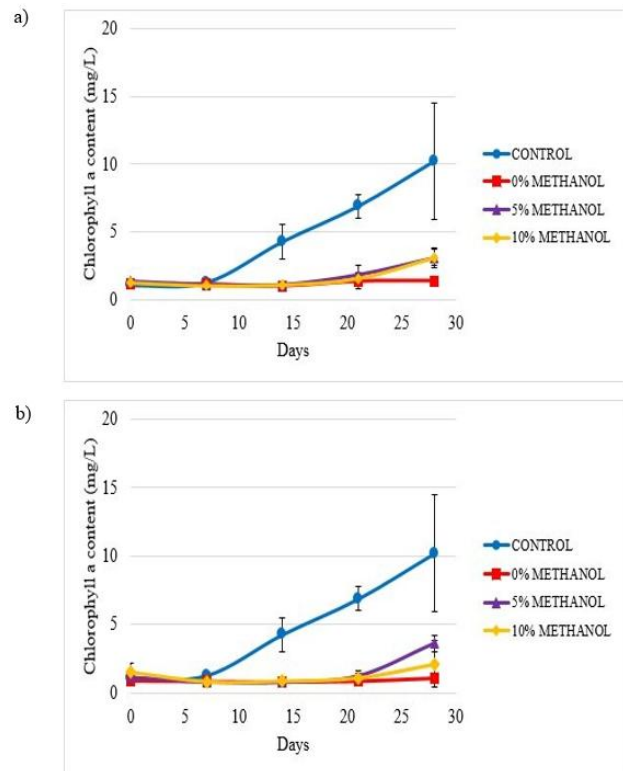


Figure 3: Growth of heterocystous cyanobacterial species: a) *Scytonema* sp. U-3-3 after 1 month of cryopreservation, b) *Scytonema* sp. U-3-3 after 2 months of cryopreservation.

Morphological appearances were also noted during the recovery process. Initially, green colonies appeared when the cryopreserved strains were inoculated into culture media after the thawing (Figure 4). After several days, these colonies turned white and settled at the bottom of the universal bottle during the lag phase. Subsequently, new green spots emerged within the white colonies, gradually forming new green colonies as culturing progressed. This result suggests that post-thaw viability at inoculation resulted from the growth of surviving cells among dead cells.

Cryopreservation is widely regarded as an effective approach for the long-term storage of microorganisms, including cyanobacteria; however, its responses can vary greatly across different species and morphological forms (Gaget *et al.*, 2017), as shown from the results obtained in this study. From the findings, only three (*Synechococcus* sp. M1, *Nodosilinea* cf. *nodulosa* LEGE 10377, and *Oscillatoria* sp. OF9) out of the twelve frozen strains showed high viability after thawing after a period of three months of cryopreservation at low temperature. In addition, it was clear that strains frozen with a

cryoprotectant demonstrated higher viability after thawing compared to those that were frozen without cryoprotectant. Methanol cryoprotectant generally enhanced post-thaw viability, with a shorter lag phase compared to those preserved without it. These results align with other studies that indicate cryoprotectants mitigate the effect of ice formation and osmotic shock, which could lead to the disruption of cellular integrity (Kapoor *et al.*, 2019). Results showed that most of the frozen strains were able to recover and show growth after some lag times. This indicates that the cryopreserved strains require time for recovery to repair the damage that resulted from the cryopreservation process. The longer the preservation time, the longer lag phase is required for recovery, which can be attributed to the increase in cell damage.



Figure 4: Recovery tests were conducted to observe the cells viability showing formation of green colonies.

From the findings, it can be inferred that the extent of cellular damage influences the duration of the lag phase (Park, 2006).

Unicellular strains such as *Synechococcus* sp. M1 proved its resistance without methanol, indicating natural tolerance to freeze stress. Notably, this strain grew faster without methanol after longer storage, which suggests the toxic effect of methanol at higher concentrations or prolonged exposure (Mazur, 1984; Rodrigues da Silva *et al.*, 2020). In contrast, *Synechococcus* sp. E068 and *Synechocystis* sp. CICALA 700 required methanol for revival, indicating inter-strain variability for the requirement of cryoprotectant. Filamentous strains showed variations in terms of their responses towards cryopreservation. *Nodosilinea cf. nodulosa* LEGE 10377 could survive even after three months regardless of cryoprotectant, suggesting strong intrinsic stress tolerance.

Meanwhile, other strains such as *Leptolyngbya* sp. D1C10 needs an optimal concentration of methanol (5%) for successful cryopreservation, while a higher concentration (10%) proved harmful, possibly due to methanol toxicity. The heterocystous cyanobacterium *Scytonema* sp. U-3-3 demonstrated low tolerance towards cryopreservation, where recovery occurs after

short-term cryopreservation with methanol and dies beyond two months. This is in contrast with a previous study, where there is a high revival rate for heterocystous cyanobacteria containing akinetes and heterocysts were recorded at low temperatures compared to vegetative cells (Watanabe and Sawaguchi, 1995). Our results suggest that the heterocystous cyanobacterium used in this study may be more vulnerable to freezing damage, probably due to its complex cell differentiation and nitrogen-fixing structures.

Low-temperature preservation is more practical compared to serial transfer since it requires very minimal equipment where only a -20 °C freezer is needed. However, the variability in responses towards cryopreservation restricts the development of a universal cryopreservation protocol for toxin-producing cyanobacteria. Several key parameters involved in cryopreservation, such as the type and concentration of cryoprotectant, thawing procedure, and preservation duration, need further optimization to be able to develop an effective and efficient long-term preservation strategy for toxin-producing cyanobacterial strains.

CONCLUSION

This work has shown that cryopreservation can be considered as an efficient approach for preservation of toxin-producing cyanobacteria, with methanol, can be successfully used as a cryoprotectant for some strains. Responses to the preservation at a lower temperature with or without cryoprotectants showed variable outcomes, indicating the lack of universality in this approach. Methanol generally enhanced post-thaw viability and reduced lag phases, but higher concentrations or longer exposure may pose harmful effects to the strains. These results emphasize the requirement to optimize critical cryopreservation parameters to develop a reliable and efficient long-term preservation strategy for diverse cyanobacterial strains.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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