I Am Cool: A study of cryopreservation in cyanobacteria.

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INTRODUCTION:

Cryopreservation, the process by which cells are preserved by cooling to sub-zero temperatures, provides a prolonged, efficient, and effective storage option for cells within research environments. Also, cryopreservation is incredibly important within medicine, providing greater patient access, manufacturability, and product safety and quality [1]. However, cryopreservation may cause damage to cells during the freezing stage [2,3]. Identification of specific strains of cyanobacteria that can be cryopreserved with high viability would be beneficial for biotechnology work. Dr. Stephen Hawley is carrying out a research program that will address the economic feasibility of producing specific compounds with engineered photosynthetic bacteria. He has purified five strains of cyanobacteria from the twin cities area. The phenotypic traits of the strains are being tested for experimental tractability within biotechnology work. The specific phenotypic trait tested within this project is recovery after cryopreservation using two different cryopreservatives: DMSO and glycerol. The five isolated cyanobacteria strains are tested using basic laboratory cryopreservation methods to identify which cryopreservative leads to the greatest revival for each strain after freezing [2].

STUDY DESIGN:

This study aimed to determine which cryopreservative (DMSO or glycerol) leads to the greatest revival after freezing for each cyanobacteria strain (sls004, sls005, sls006, sls007, sls008). In order to identify which cryopreservative led to the greatest revival for each strain, colony counts for each strain - using both cryopreservatives - were obtained after at least two weeks of cryopreservation at -80'C and compared between cryopreservatives for each strain. Two different types of replicates were used within the study to improve the measurement of variation: biological and technical. Three biological replicates of each strain were used to check for strain purity throughout cryopreservation tests. Each biological replicate had three technical replicates to check for consistency of lab technique. Final colony counts were normalized against the following variables: dilutions, amount of cryopreservatives added (differed between two cryopreservatives), differential cell growth. T-Tests were used to identify significant differences in revival between the two cryopreservatives for each strain. T-Tests were also used to identify significant differences in revival between biological and technical replicates. Necessary data sets for experiment are listed below.

Necessary Data Sets	Purpose
Growth efficiency data for each strain; cell growth, containing cryopreservative, without freezing	Determine normal growth to compare with revival from cryopreservant; determine effects of cryopreservants without freezing
Comparison of revival between cryopreservatives (DMSO and glycerol) for cyanobacteria strains	Overall effect of each cryopreservant on cyanobacteria; overall effect of each cryopreservant on each strain
Comparison of revival between cyanobacteria biological replicates for each strain for each cryopreservative (DMSO and glycerol)	Overall effect of each cryopreservant on each biological replicate; determine strain purity
Revival of *E. coli* using cryopreservants	Control for glycerol
Revival of *R. palustris* using cryopreservants	Control for DMSO

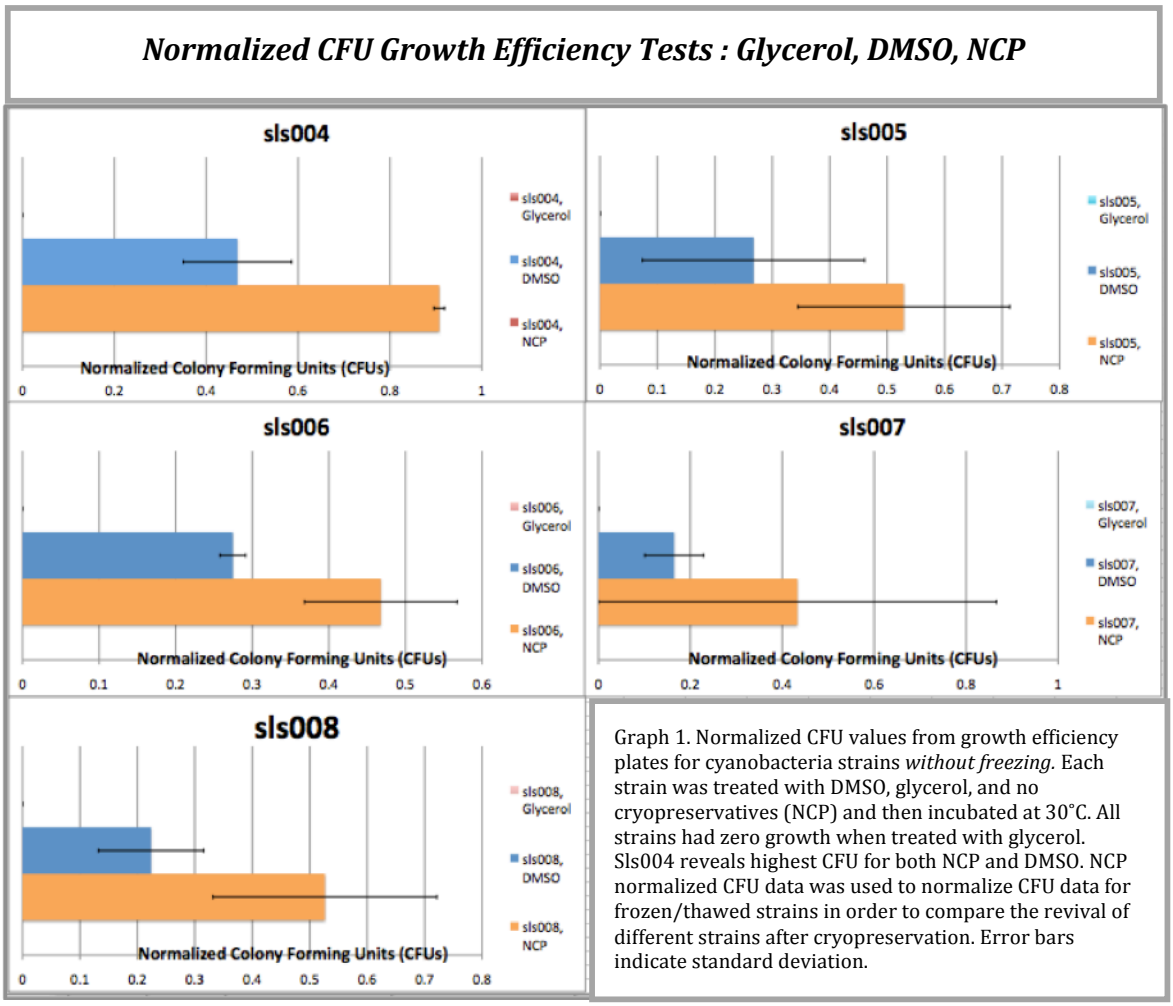
RESULTS:

Growth Efficiency Plates (no freezing): E. coli and *R. palustris* both exhibited consistent, excessive growth on their agar plates containing DMSO, glycerol, and no cryopreservative (NCP) (Table 1). All cyanobacteria strains revealed zero growth when treated with glycerol (Graph 1). sls004 revealed the greatest amount of colony forming units (CFUs) for both NCP and DMSO (Graph 1). T-tests for the growth efficiency plates for cyanobacteria strains revealed that addition of cryopreservative did not have significant effects on non-frozen cells for strains sls005 and sls008 (ttests revealed $p>0.05$). Glycerol negatively affects growth of sls004 when not frozen (ttest[sls004:glycerol, NCP]=0.011)[1]. Glycerol has a greater negative effect on sls006 than DMSO when not frozen (ttest[sls006:dmso, glycerol]=0.027). Glycerol negatively effects growth of sls007 when not frozen (ttest[sls007:glycerol, NCP]=0.019).

Table 1. Growth efficiency plates for *E. coli* and *R. palustris* display consistent, significant growth for each strain for each treatment.

Strain	Cryopreservative	Approximate Plate Coverage	Spread description
E. coli K12	NCP	90%	Lawn, even spread
E. coli K12	DMSO	90%	Lawn, even spread
E. coli K12	Glycerol	90%	Lawn, even spread
R. palustris	NCP	60%	Even spread
R. palustris	DMSO	60%	Even spread
R. palustris	Glycerol	60%	Even spread

[1]Ttest Notation: Used to determine p value for comparisons between two different cryopreservatives used on a bacterial strain: (ttest[STRAIN:CRYOPRESERVANT 1, CRYOPRESERVANT2]=P VALUE). Also used to determine p values for comparisons between biological replicates that used the same cryopreservant: (ttest[STRAIN: BIOLOGICAL REPLICATE#, #]=P VALUE).

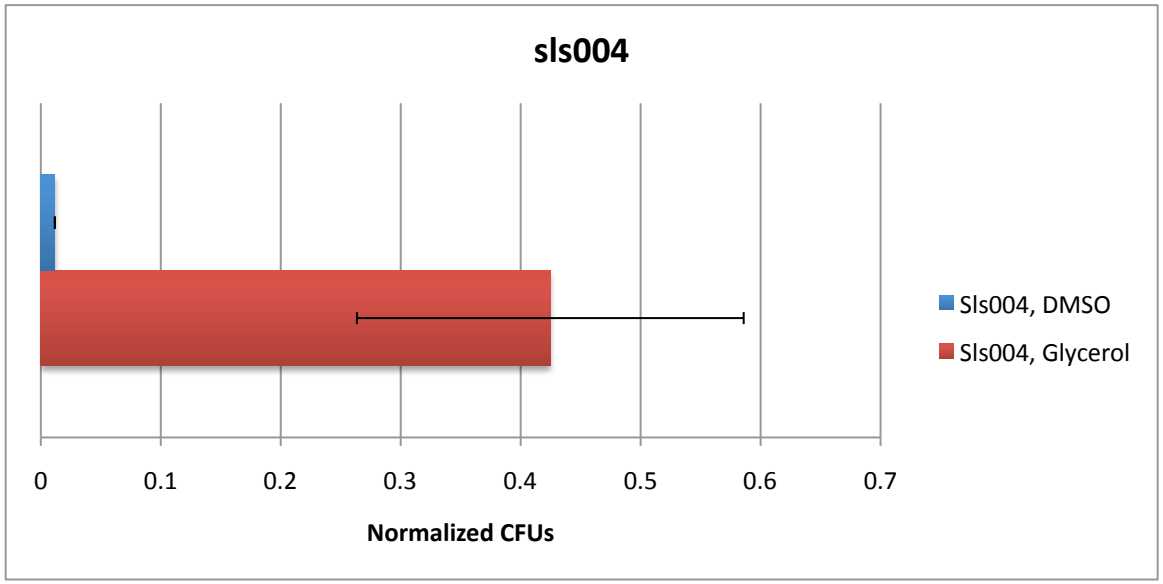


Graph 1. Normalized CFU values from growth efficiency plates for cyanobacteria strains *without freezing.* Each strain was treated with DMSO, glycerol, and no cryopreservatives (NCP) and then incubated at 30˚C. All strains had zero growth when treated with glycerol. Sls004 reveals highest CFU for both NCP and DMSO. NCP normalized CFU data was used to normalize CFU data for frozen/thawed strains in order to compare the revival of different strains after cryopreservation. Error bars indicate standard deviation.

Freezing/Thawing: Results indicated if a significant difference in strain revival existed between DMSO and glycerol treated cyanobacteria after frozen at -80˚C for two weeks. Results revealed a significant difference in revival between cryopreservatives for sls004: glycerol leads to greater cell revival than DMSO after cryopreservation (ttest[sls004:dmso,glycerol]=.008). Results also revealed a significant difference in revival between the sls004 biological replicates treated with glycerol (ttest[sls004-glycerol:reps1,2]] =0.043). Strains sls005-sls008 did not reveal a significant difference in revival between biological replicates for each cryopreservative, or between overall strain revival between cryopreservatives (ttests revealed p>0.05). Normalized CFU for biological replicates treated with both cryopreservatives reveal consistently greater revival for biological replicate 2 in strains slsoo4-sls007 (Table 3,4).

Table 2. Mean Strain CFUs using DMSO and Glycerol during Freeze/Thaw. Standard deviations of CFU values were compared; statistically significant differences are defined as overlapping standard deviation values. CFU values for Sls004 revealed a statistically significant difference in revival between the two cryopreservatives, with glycerol leading to greater revival. All other strains do not reveal a statistically significant difference in revival between cryopreservatives when standard deviations are compared.

Mean Strain CFU: DMSO vs Glycerol with Freeze/Thaw				
Strain	Normalized CFU using DMSO	Normalized CFU using Glycerol	p-value from T-Test	Statistically significant difference between cryopreservatives (Yes/No)
sls004	0.0117	0.4247	0.0081	Yes
sls005	0.3326	0.4750	0.3035	No
sls006	0.4601	0.4470	0.9441	No
sls007	0.5284	0.4047	0.4327	No
sls008	0.5814	0.8794	0.1861	No



Graph 2. Normalied CFU values for cyanobacteria strain sls004 *after freeze/thaw* using DMSO and Glycerol as cryopreservatives. Error bars indicate standard deviation and reveal a significant difference in revival between DMSO and glycerol, with glycerol leading to greater revival.

Table 3. Normalized CFU values after freeze/thaw for Glycerol Biological Replicates. Strain purity was tested by checking for significant differences in revival among replicates. Standard deviations of CFU values were compared; statistically significant differences are defined as overlapping standard deviation values. A significant difference in revival between sls006 and sls008 biological replicates was observed – with sls006 Replicate 2, and sls008 Replicate 1 obtaining greater revival. All other strains do not reveal a statistically significant difference in revival between biological replicates when standard deviations are compared.

Normalized CFUs : Freeze/Thaw : Glycerol Biological Replicates			
Strain	Normalized CFU: Biological Replicate 1	Normalized CFU: Biological Replicate 2	Statistically significant difference between biological replicates (Yes/No)
sls004	0.5387	0.3107	No
sls005	0.5459	0.4041	No
sls006	0.7091	0.1850	Yes
sls007	0.5242	0.2851	No
sls008	0.6401	1.119	Yes

Table 4. Normalized CFU values after freeze/thaw for DMSO Biological Replicates. Strain purity was tested by checking for significant differences in revival among replicates. Standard deviations of CFU values were compared; statistically significant differences are defined as overlapping standard deviation values. A significant difference in revival between sls006 biological replicates was observed – with sls006 Replicate 2 obtaining greater revival. All other strains do not reveal a statistically significant difference in revival between biological replicates when standard deviations are compared.

Normalized CFUs : Freeze/Thaw : DMSO Biological Replicates			
Strain	Normalized CFU: Biological Replicate 1	Normalized CFU: Biological Replicate 2	Statistically significant difference between biological replicates (Yes/No)
sls004	0.0117	0.0117	No
sls005	0.4184	0.2468	No
sls006	0.5843	0.3360	Yes
sls007	0.6075	0.4494	No
sls008	0.5726	0.5903	No

DISCUSSION:

Effects of cryopreservatives on growth efficiency plates (without freezing)

Results from the DMSO growth efficiency plates for sls005 and sls007 indicate that DMSO does not have a negative effect on the growth of these strains when not frozen. This response is phenotypically favorable because it indicates an experimentally tractable trait in each strain – the ability to tolerate cryopreservatives without freezing. However, sls004, ss006, and sls008 revealed significant differences between cryopreservative treatments with no freezing, which can be seen in the non-overlapping significance intervals in Graph 1.

All growth efficiency plates for the cyanobacteria strains + glycerol revealed zero growth. This response could signify that glycerol is extremely toxic for the cyanobacteria strains. However, it would be worthwhile to re-test the glycerol/no-freezing treatment for each of the strains to confirm that glycerol is toxic to the strains when not frozen and that the glycerol was not incidentally toxic for this particular experiment.

R. Palustris and *E. Coli* were used as controls and had consistent, sufficient growth, tolerating both cryopreservatives without freezing. These results support that the cryopreservatives were not contaminated and were well tolerated by both control strains without freezing.

Freeze/thaw comparisons

Results from the freeze/thaw comparison between DMSO and glycerol for strains sls005 – sls008 had p-values > 0.05. This indicates that there is a significant probability that the differences in revival between DMSO and glycerol were due to chance. However, due to the small sample size, meaningful conclusions cannot be drawn by this data. The results suggest that cryopreservatives DMSO and glycerol can both be successfully used for freezing strains sls005 – sls008. However, a larger sample size is needed to further support these results and detect if a statistically significant difference actually exists.

The p values for the biological replicates within each cryopreservative treatment for sls004 – sls008 (treated with DMSO) indicate strain purity and validates the precision and accuracy of the data (Values for biological replicates >0.05 – indicating no significant difference). Sls004 reveals a p-value <0.05 for glycerol/DMSO comparison, indicating that sls004 has significantly greater revival from cryopreservation using glycerol. This data indicates a phenotypically favorable trait. It would be advantageous to use glycerol as a cryopreservative over DMSO for cyanobacteria because it is less toxic and less expensive. However, this data will need to be further verified due to the uncertainty of the purity of sls004, indicated by the p-value>0.05 for sls004 treated with glycerol.

It should be noted while interpreting this data that a very small sample size was used. Therefore, it is possible that the conclusions from the results have a large degree of variability and future observations could be significantly different.

CONCLUSION:

The purpose of this investigation was to identify which cryopreservative (DMSO or glycerol) leads to the greatest cryopreservation revival for each cyanobacteria strain (sls004, sls005, sls006, sls007, and sls008). The study did not detect a significant difference in cryopreservation revival for strains sls005-sls008. Strain sls004 revealed greater revival after cryopreservation with the use of glycerol.

For further investigation on this project, it would be worthwhile to repeat the experiment with a larger sample size for each cyanobacteria strain. A larger sample size is needed to further support the results and have the statistical power to detect if a statistically significant difference actually exists between cryopreservatives DMSO and glycerol. This would also confirm whether or not sls004 has greater revival after freezing using glycerol over DMSO. If confirmed, this trait would be unique within the phylum of cyanobacteria, which normally recover more effectively from cryopreservation using DMSO [4]. Also, more aspects of cryopreservation viability will need to be carried out in order to gain greater confidence in the phenotypic favorability of the specific strains. Testing for metabolic activity would be an appropriate next step in assessing the strain's recovery from freezing. In the future, other tests on phenotypic characteristics would lead to greater knowledge of potential strains for use in biotechnology.

In a broader perspective, preliminary data was obtained for identifying phenotypic traits that are accommodating for biotechnology research. The identification of experimentally tractable traits will contribute to addressing the economic feasibility of using engineered photosynthetic bacteria to produce useful compounds [5]. Applications of this technology have the possibility of creating a distributed bioprocess industry, which would reduce the transportation costs and match the environmental costs to the economic benefits of production in a community (specifically communities in the developing world)

METHODS:

Organisms: Five strains of cyanobacteria were obtained from Dr. Stephen Hawley's lab and studied for recovery after cryopreservation: sls004, sls005, sls006, sls007, sls008. 18mm BG-11 tubes were inoculated with single colonies from cyanobacteria strain streak plates grown under fluorescent lamps on in an incubator at 30˚C. *Escherichia coli* K12 mg1655 and *Rhodopseudomonas palustris* CGA009 were used as control strains. *E. coli* was grown up in M9 liquid medium at 30˚C. *R. palustris* was grown up on PM liquid medium at 30˚C.

Strain	*Description*	*Media Used*	*Source*
sls004*	Cyanobacteria, green; environmental sample, enrichment for photoautotrophic organisms	BG-11 medium	Dr. Hawley's Lab (UMN)
sls005*	Cyanobacteria, green; environmental sample, enrichment for photoautotrophic organisms	BG-11 medium	Dr. Hawley's Lab (UMN)
sls006*	Cyanobacteria, green; environmental sample, enrichment for photoautotrophic organisms	BG-11 medium	Dr. Hawley's Lab (UMN)
sls007*	Cyanobacteria, green; environmental sample, enrichment for photoautotrophic organisms	BG-11 medium	Dr. Hawley's Lab (UMN)
sls008*	Cyanobacteria, green; environmental sample, enrichment for photoautotrophic organisms	BG-11 medium	Dr. Hawley's Lab (UMN)
Escherichia coli K12 mg1655 [6]	Bacteria, white; Laboratory strain with minimal genetic manipulation	M9 medium	Dr. Gralnik's Lab (UMN)
Rhodopseudomonas palustris CGA009 [7]	Bacteria, purple; Laboratory strain	PM medium	Dr. Hawley's Lab (UMN)

*First study on this particular strain

Culture media:

1. BG-11 was used as growth media for cyanobacteria. BG-11 agar plates contained 0.05% Thiosulfate [8].
2. M9 liquid medium was used as a basic experimental solution for *E. coli.* The preparation of 200mL M9 liquid medium included the following: Stock 1 was prepared was prepared in the following way: Stock 1 was prepared by adding 128g/L Na_2HPO_4 * 7 H_20, 30g KH_2PO_4, and 5g NaCl to 1 L of ddH_20. To prepare 200mL M9 media, 20ml Stock 1, 400ul $MgSO_4$ (1M), and 2ml 10% NH_4Cl were brought to 200mL with ddH_20. Media was autoclaved and cooled. The autoclaved liquid was then combined with 200ul of 100mM $CaCl_2$, 200ul of 10mM $FeSO_4$, and 4ml of 20% glucose.
3. Photosynthetic Medium (PM) liquid medium was used as a basic experimental solution for *R. palustris.* The preparation of PM medium included the following: 25ml 0.5 M Na_2HPO_4, 25ml 0.5 M KH_2PO_4, 10ml 10% $(NH_4)_2SO_4$, 1ml Concentrated base, 1ml 0.1 sodium thiosulfate, and 1ml 2mg/ml stock p-aminobenzoic acid were added to 1 L ddH_2O. Medium was autoclaved. 10mM sodium bicarbonate and 10mM anaerobic succinate were added to media after autoclaving.
    a. Concentrated base solution contained (per liter) 20g Nitrilotriacetic acid, 28.9g $MgSO_4$ anhydrous, 6.67g $CaCl_2$ * 2 H_2O, 18.5mg, $(NH_4)_6Mo_7O_{24}$ * H_2O, 198mg $FeSO_4$ * 7 H_2O, and 100ml metal 44 solution. Final pH was adjusted to 6.8. Media was stored at 4'C.
    b. Metal 44 Solution contained (per liter) 2.5g EDTA, 10.95g $ZnSO_4$ * $7H_2O$, 5.0g $FeSO_4$ *7 H_2O, 1.54g $MnSO_4$ * H_2O, 392mg $CuSO_4$ * 5 H_2O, 250mg $Co(NO_3)_2$ * 6

H_2O, and 177mg $Na_2B_4O_7$ * 10 H_2O. A few drops of concentrated H_2SO_4 were added to retard precipitation. Solution was protected from light with aluminum foil.

4. Dimethyl sulfoxide (DMSO) and glycerol (50%) were used as cryopreservatives. The cryopreservatives were incorporated into the basic experimental solutions when used. DMSO and glycerol were sterile via filter sterilization and autoclave accordingly. The cryopreservatives were obtained from the Hawley Laboratory.

Calculating growth efficiency: Cells were used directly from 10ml liquid cultures grown up to an OD of 0.4 (Cyanobacteria measured at OD730, *R. Palustsris* measured at OD660, *E. coli* measured at OD600). ~9ml of liquid cultures were centrifuged and re-suspended in 1ml liquid culture to create 10X liquid cultures (liquid cultures are reduced from 10ml (initial volume) to 9ml after autoclaving and cell growth). Cells were diluted and then exposed to DMSO, glycerol, and no cryopreservative (ncp). Cells, treated in three different ways, were then plated on their indicated basic solution agar plates. Plates were covered in cheesecloth and grown up under fluorescent light at 30°C. Colony forming units (CFU) were counted on each of the plates after sufficient growth using the following method: each plate was photographed and uploaded to the ImageJ software. The "Cell Counter" plugin was used to quantify the number of cell colonies: each colony was selected on the screen (identified by the human counter to ensure that colonies close to one another were differentiated and accounted for) and the plugin kept track of counted colonies. This method ensured that all colonies were identified and accounted for (by a human counter that can recognize the difference between multiple colonies), and that colony count was kept track of in an accurate manner (by the plugin).

Freezing and thawing: Cells were used directly from 10ml liquid cultures grown up to an OD of 0.4. 10ml of liquid cultures were centrifuged and re-suspended in 1ml liquid culture to create 10X liquid cultures. Cells were exposed to cryopreservatives (DMSO treated cells: 24ul DMSO : 300ul cell culture; Glycerol treated cells: 1ml 50% glycerol : 1ml cell culture) and immediately put on ice for up to 15 minutes. After 15 minutes, cells were further diluted to a concentration of 1:5000 to allow for cell counting after revival. Cells were then frozen at -80°C for two weeks minimum. After the two-week freezing period, cells were thawed on ice, diluted, and plated on their indicated basic solution agar plates. Plates were covered in cheesecloth and grown up under fluorescent light at 30°C.

Measuring viability:
Viability after cryopreservation for each strain (using DMSO and glycerol) was compared among strains and between cryopreservatives for each strain using calculated "Normalized Colony Forming Units" (CFUs). The Normalized CFUs for each strain were calculated in the following manner: cell colonies were counted on the plate manually and verified by ImageJ [9]; the number of counted colonies was multiplied by the plating dilution factor (5000); the cell culture composition (cell culture/total culture volume including cryopreservative) before frozen was accounted for; finally, the value was divided by the initial cell count for each culture (as calculated by culture sample plated with no cryopreservative and no freezing). CFUs are used as the comparison for cell viability between strains and within strains using different cryopreservatives. "Raw data" refers to number of colonies formed after cryopreservation before normalization of data. Counting the number of CFUs on the plates and comparing normalized CFU data assessed cell recovery after cryopreservation.

Equation derivation for normalized CFU count:
The CFUs are normalized in order to compare the cell counts between strains using same and different cryopreservatives. The number of colonies counted on the plate after recovery is multiplied by the dilution factor (5000) to reach the initial amount of cells plated; it is also multiplied by the percent liquid cell culture in the mixture of cell culture + cryopreservative (0.9999 or 0.5) to account for the volumes of differing cryopreservatives. This product is then divided by the initial cell count to account for initial small differences in cell OD.

Normalized CFU Equations:

n=f(c)

f(c)= (cdp)/i

n = normalized CFU
c = colony count
d = dilution factor
p = % cell culture within culture+cryopreservative mixture; Parameter values: 0.9999 for glycerol, 0.5 for DMSO
i = initial cell count on plates after revival from cryopreservation

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SOURCES:

1. Hubel, Allison. "Emerging issues in cryopreservation of cellular therapies." BioCor: Advancing the science, technology and practice of bio-preservation. n.d. Web. 1 Mar. 2011.

2. Day JG. 2007. Cryopreservation of microalgae and cyanobacteria. Methods of Mol Biol. 368: 141-51. Review.

3. Day, JG; Watanabe, MM; and Turner, MF. 1998. Ex-situ conservation of protistan and cyanobacterial biodiversity: CCAP-NIES collaboration 1991–1997. Phycological Research, 46: 77–83.

4. Wantanabe M and Sawaguchi T. 1995. Cryopreservation of a water-bloom forming cyanobacterium, *Microcystisaeruginosa f. aeruginosa. Phycological Research.* National Institute for Environmental Studies, 16-2 Onogawa, Tuskuba, Ibaraki 305, Japan. 43:111-116.

5. Rupprecht J et al. 2006. Perspectives and advances of biological H2 production in microorganisms. Applied Microbiology and Biotechnology. 72, 3:442-449. Review.

6. Blattner F et al. 1997. The Complete Genome Sequence of Escherichia coli K-12. *Science,* New Series, 277(5331): 1453-1469.

7. Nordberg G et al. The Genome Portal of the Department of Energy Joint Genome Institute. Nucleic Acids Res 2011 0: gkr947v1-gkr947

8. Rippka R 1988. Isolation and purification of cyanobacteria. *Methods Enzymol* 167:3–27.

9. Abramoff M, et al. 2004. Image processing with ImageJ. *Biophotonics International.* 11(7): 36-42.