THE CANADIAN SCIENCE FAIR JOURNAL

ARTICLE

Biograde Yeast: Biomanufacturing with Antifreeze Yeast on Mars

Patricia Rea

15| Markham, Ontario

1st Place - 2019 Lunenfeld-Tannenbaum Research Institute Science Fair | Gold Medalist, York Region Science & Technology Fair 2019 | Canada Wide Science Fair 2019 Excellence in Astronomy Award | Canada-Wide Science Fair 2019 Silver Medal | Team Canada - MAGMA Exporecerca Jove International Research Fair 2020 (Barcelona) | International IARC Young Scientist Award - MAGMA Exporecerca Jove International Research Fair 2020

The Artemis missions are planning to have humans on the Moon by 2024 and will act as a steppingstone for getting to Mars (10). Long-term settlements in space will have to be self-sufficient, producing their own resources, while keeping in mind transport constraints. Yeast is a perfect candidate for use on these missions as it is relatively resilient, well understood, and it is small and lightweight. It is an effective biofactory and can produce biodegradable plastic, spider silk, and milk (1-4). But if there was an environmental failure within the colonist's developed biosphere, and the yeast samples were exposed to the extreme environmental conditions that exist on the Moon (-248°C to 123°C) or Mars (-140°C to 30°C, with an average of -63°C), most of the samples would die, which could be catastrophic for colonists (11, 12). This research is part of the development of a strain of *Saccharomyces cerevisiae* (*S. cerevisiae*) with the goal of surviving these temperatures through the use of antifreeze proteins (AFPs). A genetically modified strain of *S. cerevisiae* capable of surviving cold conditions could give long term exploration of terrestrial and extraterrestrial areas access to a hardier material and food supply.

INTRODUCTION

Ice binding proteins are typically small proteins with the ability to absorb to the surface of ice crystals (6, 7). AFPs, a subclass of ice binding protein, are able to inhibit the growth of ice crystals through an absorption-inhibition mechanism. These proteins are thought to interact with water molecules and organise them into an ice-like lattice on the ice-binding site, then absorb onto the quasi-liquid layer of ice, freezing the AFP onto the crystal. This causes a micro-curvature of the surface of the crystal, which makes it thermodynamically difficult for water molecules to freeze onto the surface and allow for the ice crystal to increase in size. This results in a depression of the freezing point, as well as a slight increase in the melting point, which is known as thermal hysteresis. The difference between the melting and freezing point is known as the thermal hysteresis gap (7, 8). AFPs from different organisms vary in activities, insect AFPs being generally the most active (9).

The cfAFP-501 isoform is a hyperactive β-solenoid AFP originating from Choristoneura fumiferana (spruce budworm) (Figure 1). Zvafp13 is a moderately active globular AFP which originates from Zoarces viviparus (viviparous eelpout) (Figure 2). In this research, the Zvafp13 and cfAFP-501 genes were inserted into plasmids (circular strands of DNA) and, through heating of the yeast-membrane, were separately introduced into *S. cerevisiae*. The samples were then frozen at -30°C, -80°C, and -196°C for 3 hours, 24 hours, 7 days, and 28 days, with the goal of increasing the survival of the engineered *S. cerevisiae* as a result of the addition of the AFPs. This research builds on work conducted in 2018, where *S. cerevisiae* was engineered with green fluorescent protein (GFP) and tested for survival in varying temperatures.

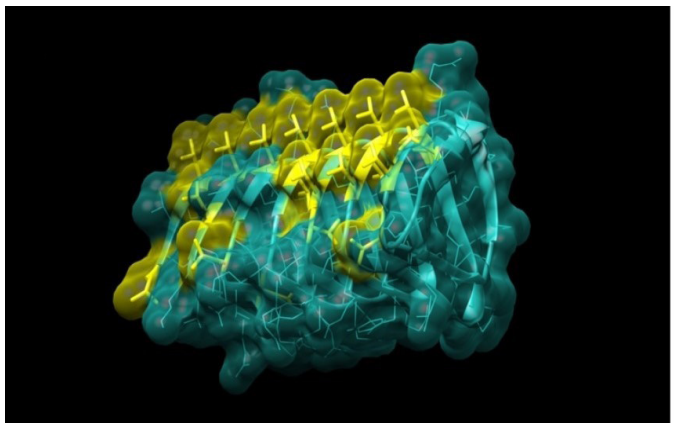

Figure 1. cfAFP-501 Protein Structure. (Protein Data Bank code 1m8n) protein structure (left). Yellow indicates the amino acid Threonine (which plays a key role in the function of the cfAFP-501) in the ice binding site. The image in this figure was generated using Chimera.

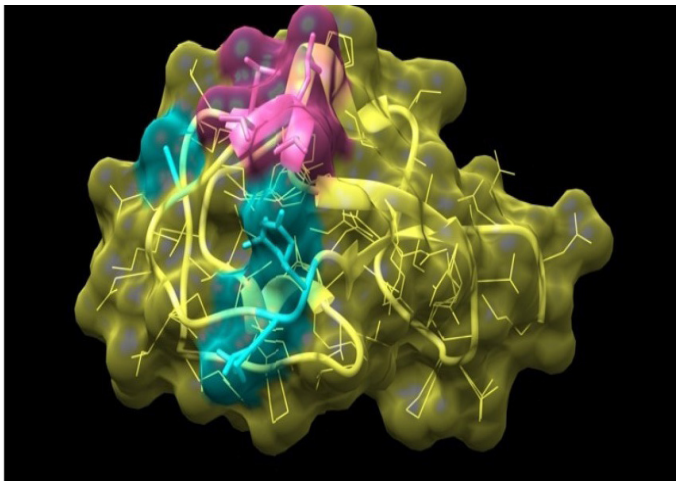

Figure 2. Zvafp13 Protein Structure. (Protein Data Bank code 4ur4) protein structure (right). Pink indicates the key amino acids in the primary prism plane ice binding site, while teal indicates the key amino acids in the pyramidal plane ice binding site (13). The image in this figure was generated using Chimera.

METHODS

Transformation of S. cerevisiae with Plasmid DNA

1-2uL of *S. cerevisiae* was suspended in 100uL of yeast transformation mix (100mM Lithium Acetate, 40% PEG 8000, 0.1mg/mL Salmon Sperm DNA) at 4°C. 10uL of plasmid DNA was added per sample and incubated at 42°C for an hour, after which the samples were incubated at room temperature overnight before plating.

Expression of AFPs in S. cerevisiae

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the expression of the plasmid in engineered *S. cerevisiae* samples. It is important to note that there is no data for the cfAFP-501-engineered *S. cerevisiae*, as it had not been successfully engineered at the time the SDS-PAGE gels were run. The TEF1 promoter was used in all plasmids.

Freeze Testing

Engineering of the cells was conducted using plasmids with G418 resistance. The AFPs expressed were Zvafp13 and cfAFP-501, while the ASTU and RFP proteins acted as negative controls. The ASTU protein was used to confirm that any survival advantage was not caused by the selection marker, and the RFP protein was used to confirm that any survival advantage was not caused by the introduction of a non-native protein.

All samples were tested at -30°C, -80°C, and -196°C (liquid nitrogen). Liquid nitrogen samples were transferred to Cryotubes, while the rest of the samples were in regular microcentrifuge tubes. Samples were frozen for 3 hours, 24 hours, 7 days, and 28 days to see how long engineered yeast, both with and without AFPs, could survive in cold conditions. Testing in each time and temperature zone was conducted in duplicate. Survival was measured against control samples stored at 4°C for 7 days, as drying issues with some 3 hour and 24-hour samples made this the most reliable control for comparison. In some cases, this results in survival percentages exceeding 100%. Average cell survival count per tube was found, then standard deviation and standard error of the mean was applied. Only samples with a minimum of 2 colonies were used, any sample with 1 colony was removed.

RESULTS

SDS-PAGE results

SDS-PAGE results (Figure 3) showed faint lines underneath both Zvafp13 samples at the proper kDa level (7.7kDa) for the protein, suggesting the expression of the Zvafp13.

cfAFP-501-engineered S. cerevisiae survival

cfAFP-501-engineered *S. cerevisiae* at -30°C demonstrates a 3-hour survival advantage, the survival percentage at 3 hours was 100.12% (error bars go over 125.0% and under 75.0%), while at 24 hours this was reduced to 43.99% (Figure 4A).

The cfAFP-501-engineered *S. cerevisiae* at -80°C demonstrated a 7-day survival advantage, the survival percentage at 3 hours was 197.31% (error bars range from just over 100.0% to over 275.0%), at 24 hours was 83.14% (error bars range from just over 50.0% to over 100.0%), and at 7 days was 44.23% (error bars range from just over to under 50.0%) (Figure 4B).

The cfAFP-501-engineered *S. cerevisiae* at -196°C demonstrated no survival advantage, the survival percentage was highest at 24 hours (2.77%, error bars range from just over 0.0% to over 5.0%) (Figure 4C).

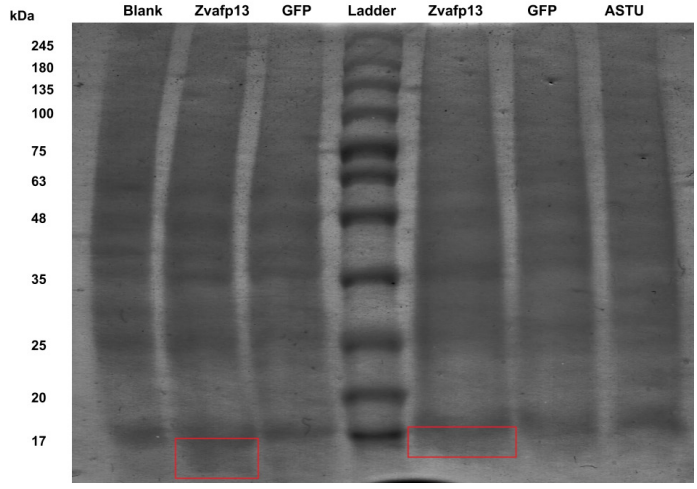

Figure 3. SDS-PAGE results. Lanes (left to right): blank *S. cerevisiae* (un-engineered), Zvafp13-engineered *S. cerevisiae*, GFP-engineered *S. cerevisiae*, protein size (kDa) standard ladder, Zvafp13-engineered *S. cerevisiae*, GFP-engineered *S. cerevisiae*, ASTU-engineered *S. cerevisiae*. Red boxes indicate the suggested expression of the Zvafp13.

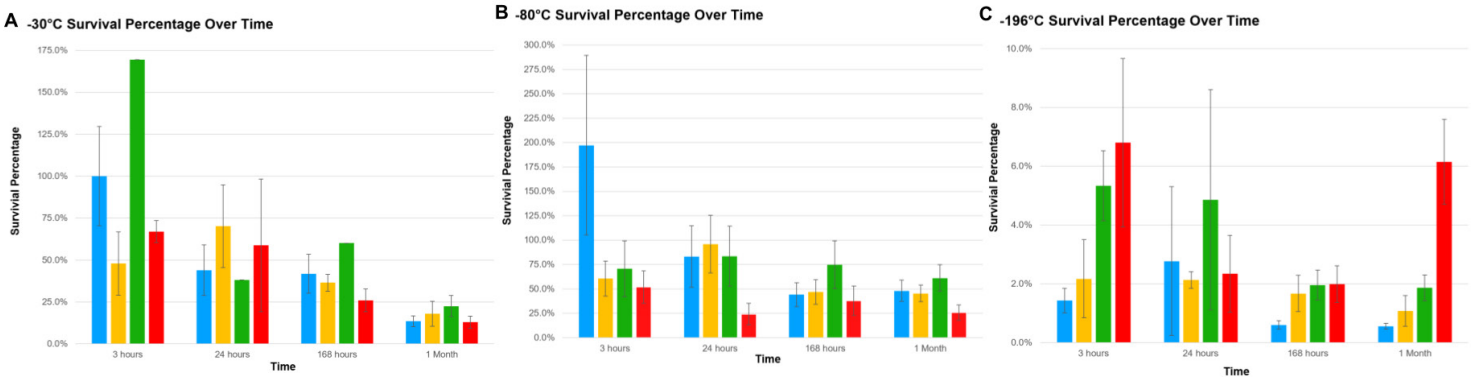

Figure 4. Survival percentage data comparison. Blue (cfAFP-501-engineered *S. cerevisiae*), yellow (Zvafp13-engineered S. *cerevisiae*), green (ASTU-engineered *S. cerevisiae*), and red (RFP-engineered *S. cerevisiae*). (A) Survival data comparison for -30°C percentiles with error bars. (B) Survival data comparison for -80°C percentiles with error bars. (C) Survival data comparison for -196°C percentiles with error bars.

Zvafp13-engineered S. cerevisiae

The Zvafp13-engineered *S. cerevisiae* demonstrated a long term survival advantage at -30°C, -80°C, and -196°C, the survival percentage was the greatest at 24 hours (70.20% for -30°C, error bars ranging from just under 50.0% to just under 100.0%, 95.83% for -80°C, error bars ranging from under 75.0% to ~125.0%) (Figures 4A, 4B) and 3 hours (2.17%, error bars range from ~1.0% to over 3.0%) (Figure 4C), respectively.

DISCUSSION

Survival percentage analysis

For Zvafp13-engineered *S. cerevisiae*, there is a noticeable pattern in the general survival percentage at -30°C and -80°C, there was a peak seen in survival at 24 hours, the second highest survival was at 3 hours, then 7 days and 28 days. The only temperature where this differs is at -196°C, where the highest survival was at 3 hours, then 24 hours, 7 days, and 28 days. This change in survival pattern at -196°C could be because species express moderately active Type III AFPs at much higher temperatures than -196°C (5).

For cfAFP-501-engineered *S. cerevisiae*, a general decrease in survival was seen over time, with two exceptions: at -80°C, a slight improvement in survival was seen between 7 days and 28 days; and at -196°C, the greatest survival percentage was achieved at 24 hours. When all data is compared on the same scale, there is an apparent survival peak at -80°C, with a severe drop in overall survival at -196°C.

CONCLUSION

Through analysis of the SDS PAGE gels, there is a likelihood that the Zvafp13 is being expressed in *S. cerevisiae*, while cfAFP-501 expression has not yet been determined. The -30ºC and -80ºC freezing results are suggestive of a possible short-term survival advantage for the cfAFP-501-engineered *S. cerevisiae* in colder temperatures of 24 hours and 7 days respectively, though because of the small sample size further research needs to be conducted. The trendline for the Zvafp13-engineered *S. cerevisiae* has little change over time in all three temperature zones, which is suggestive of a longer-term survival advantage. Since Zvafp13 is moderately active, it is expected that the Zvafp13-engineered S. *cerevisiae* would initially have lower survival. Building on this research, work is currently being conducted to develop a yeast actin-cfAFP-501 polymer able to exceed the activity of the AFPs used in this research. *S. cerevisiae* (both with and without AFPs) could theoretically survive some of the temperatures of Mars, survival is not high enough to supply enough resources for colonists were there to be a systems failure.

ACKNOWLEDGEMENTS

I would like to thank my mentors, Dr. Justin Pahara (Co-founder of Amino Labs), who helped me with experimental outlines, taught me how to properly research and reverse engineer proteins, work with PyMOL and Chimera, conduct specific procedures (I was mentored by him through e-mentoring as he is located in Alberta), as well as Amy Yeung (President of UofT iGEM team) and Keoni Gandall (Bioengineering Research Assistant, Stanford University), for offering their time and advice. I would like to thank Dr. Robert Tsushima (Chair of Biology at York University) for offering lab/freezer space for freeze testing, Dr. Mark Bayfield and Dr. Emanuel Rosonina for their advice in analysing PAGE gels for *S. cerevisiae*, Amino Labs for their support and equipment, Dr. Peter L. Davies (Canada Research Chair in Protein Engineering, Professor, School of Medicine, Department of Biological and Molecular Studies, Queen's University) for his time and support in helping me understand the function of AFPs and IBPs, and both my mom and dad for their support both financially and through their allowing me to conduct this research.

REFERENCES

1. Tokareva, O., Michalczechen-Lacerda, V. A., Rech, E. L., & Kaplan, D. L. (2013). Recombinant DNA production of spider silk proteins. *Microbial Biotechnology, 6(6)*, 651–663. https://doi.org/10.1111/1751-7915.12081
2. Lu, W., Ness, J. E., Xie, W., Zhang, X., Minshull, J., & Gross, R. A. (2010). Biosynthesis of Monomers for Plastics from Renewable Oils. *Journal of the American Chemical Society, 132(43)*, 15451–15455. https://doi.org/10.1021/ja107707v
3. Sheikh, K. (2019, August 2). Got Impossible Milk? The Quest for Lab-Made Dairy. The New York Times. https://www.nytimes.com/2019/08/02/science/lab-grown-milk.html?0p19G=7900
4. Wohlsen, M. (2015, April 15). Cow Milk Without the Cow Is Coming to Change Food Forever. Wired. https://www.wired.com/2015/04/diy-biotech-vegan-cheese/#
5. Bar Dolev, M., Braslavsky, I., & Davies, P. L. (2016). Ice-Binding Proteins and Their Function. *Annual Review of Biochemistry, 85(1)*, 515–542. https://doi.org/10.1146/annurev-biochem-060815-014546
6. Vance, T. D. R., Bayer-Giraldi, M., Davies, P. L., & Mangiagalli, M. (2019). Ice-binding proteins and the 'domain of unknown function' 3494 family. *The FEBS Journal, 286(5)*, 855–873. https://doi.org/10.1111/febs.14764
7. Phippen, S. W., Stevens, C. A., Vance, T. D. R., King, N. P., Baker, D., & Davies, P. L. (2016). Multivalent Display of Antifreeze Proteins by Fusion to Self-Assembling Protein Cages Enhances Ice-Binding Activities. *Biochemistry, 55(49)*, 6811–6820. https://doi.org/10.1021/acs.biochem.6b00864
8. Leinala, E. K., Davies, P. L., Doucet, D., Tyshenko, M. G., Walker, V. K., & Jia, Z. (2002). A β-Helical Antifreeze Protein Isoform with Increased Activity STRUCTURAL AND FUNCTIONAL INSIGHTS. *Journal of Biological Chemistry, 277(36)*, 33349–33352. https://doi.org/10.1074/jbc.M205575200
9. Graether, S. P., Kuiper, M. J., Gagné, S. M., Walker, V. K., Jia, Z., Sykes, B. D., & Davies, P. L. (2000). β-Helix structure and ice-binding properties of a hyperactive antifreeze protein from an insect. *Nature, 406(6793)*, 325–328. https://doi.org/10.1038/35018610
10. NASA: Artemis. (2019). NASA. https://www.nasa.gov/specials/artemis/
11. In Depth | About the Moon – Moon: NASA Science. (2018). Moon: NASA Science. https://moon.nasa.gov/about/in-depth/
12. mars.nasa.gov. (2019). Planet Mars. NASA's Mars Exploration Program. https://mars.nasa.gov/all-about-mars/facts/
13. Verreault, D., Alamdari, S., Roeters, S. J., Pandey, R., Pfaendtner, J., & Weidner, T. (2018). Ice-binding site of surface-bound type III antifreeze protein partially decoupled from water. *Physical Chemistry Chemical Physics, 20(42)*, 26926–26933. https://doi.org/10.1039/C8CP03

PATRICIA REA

Age:15 | Markham, Ontario

Patricia Rea is an independent synthetic microbiologist and student researcher working on a strain of yeast capable of surviving the conditions of Mars. She is one of the junior editors of the book Zero to Genetic Engineering Hero by Dr. Justin Pahara and Julie Legault. In 2019 she was invited to present her research at MIT as part of Biosummit 3.0. Patricia volunteers with STEM Kids Rock, a youth-led STEM outreach group in Markham, and is working to start Ontario's first high school iGEM team.

Patricia has received numerous awards for her work including: Bronze Medalist, York Region Science & Technology Fair 2018, 1st Place - 2019 Lunenfeld-Tannenbaum Research Institute Science Fair, Gold Medalist, York Region Science & Technology Fair 2019, Canada Wide Science Fair 2019 Discovery Award, Canada Wide Science Fair 2019 Excellence in Astronomy Award, Canada-Wide Science Fair 2019 Silver Medal, Team Canada - MAGMA Exporecerca Jove International Research Fair 2020 (Barcelona), International IARC Young Scientist Award - MAGMA Exporecerca Jove International Research Fair 2020, WISEST Externship Award - INSPO Research and Innovation Fair, Silver Medal - 2020 INSPO Research and Innovation Competition and the Distinguished Bioinformatics Project Award - INSPO.