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Four Points by Sheraton Denver Southeast



HOMEBREWING *with* **ALTITUDE**

Freeze Your Yeast!

Long term storage options for your
most precious strains

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Freeze your yeast

- How to successfully freeze your yeast
 - What you need
 - Where to get it
- Why it works
 - Published experimental evidence
 - My tests

How to do it

- Grow your culture to stationary phase
 - Can be straight from a smack pack or tube
- Place culture in refrigerator for 36-72 hours
- Add *cold* glycerol to 20% final concentration
 - Do not let the culture warm up
- Pour into tubes
- Put tubes in a zip top bag or use parafilm
- Put in the freezer in a frozen, insulated container

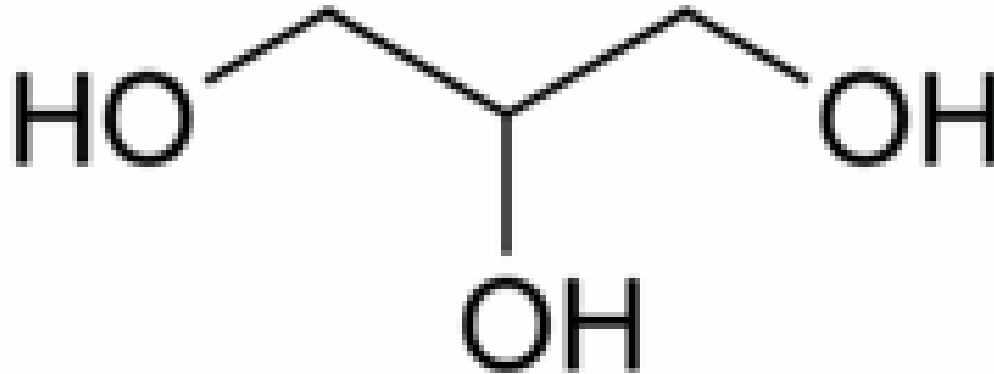
What you need and where to get it

- Insulated container
 - Six-pack cooler
 - Small styrofoam cooler
 - Insulated thermos
 - Available from any megastore, ~\$10



What you need and where to get it

- Glycerol aka glycerine
 - Available at many pharmacies
 - Used for making soap, lotions, etc



What you need and where to get it

- Tubes
 - 1.5 ml Microfuge tubes
 - 15 ml Falcon tubes
 - Anything else that can be sterilized and can handle freezing
 - Available on eBay
 - 1.5 ml tubes 50 for \$3
 - 1.5 ml tubes 100 for \$4.25
 - 15 ml tubes 50 for \$14.95



Why it works – Stationary phase

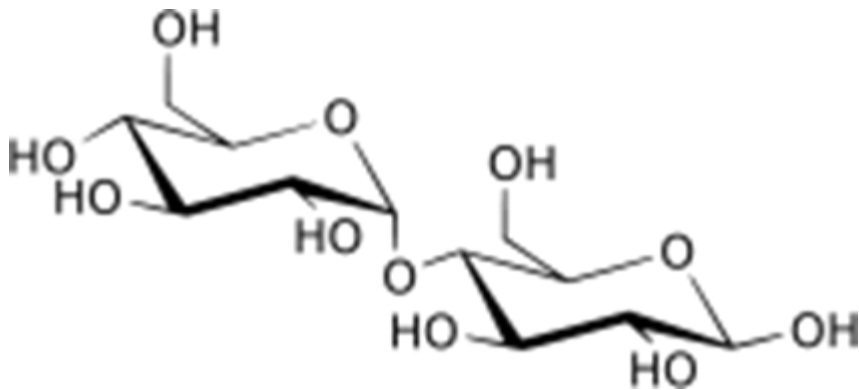
- Park *et al.* 1997
- Freeze thaw response is growth phase specific, not controlled by glucose repression
- When cells enter stationary phase, they accumulate glycogen and trehalose, develop thick cell walls, and become thermotolerant

Why it works – cryoprotectants

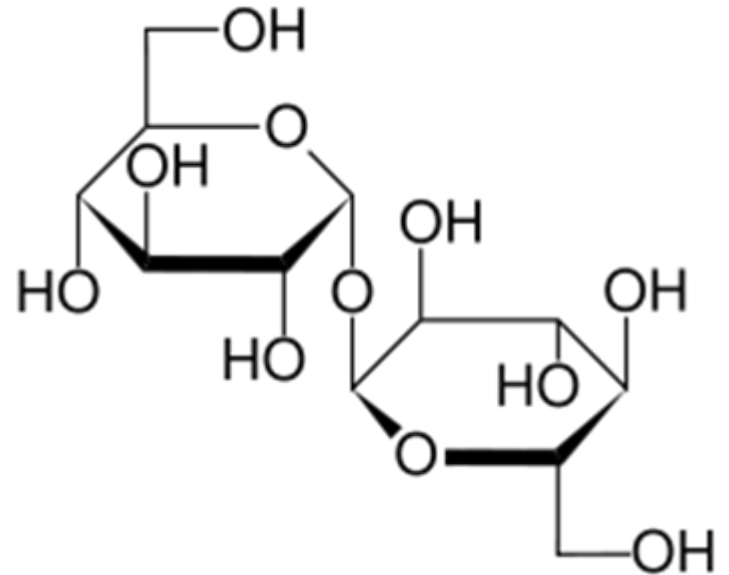
- Protect cells from damage due to freezing
 - Glycerol
 - Trehalose
 - DMSO
 - Methanol, ethanol
- In some cases it's not clear how they work
 - Bind to membranes and proteins
 - Prevent formation of large ice crystals

Why it works – trehalose

- Disaccharide made up of two glucose molecules linked via $\alpha(1-1)$ bond



Maltose



Trehalose

Why it works – trehalose

- From Kandrор *et al.* 2004
- Freeze tolerance closely correlates with cellular trehalose content
- Dramatic accumulation of trehalose and induction of trehalose synthesizing enzymes below 10°C
- After 15-20 hours at 4°C, mRNAs of trehalose synthesizing enzymes are up at least 20 fold and maintained at that level for up to 85 hours
- Longer incubation at 4°C results in an increase in cell survival (confirmed by Stoycheva *et al.* 2007)
- Cells incubated 48h at 4°C accumulated about 15-fold more trehalose compared to cells cultivated at 30°C. incubation for short periods at 4°C increases trehalose only 3-fold.
- Upon return to 30°C, mRNAs, trehalose levels, and tolerance to freezing fall dramatically within minutes

Why it works – other conditions

- Cerrutti *et al.* 2000
- Specific interactions of trehalose with membranes and/or proteins may help the freeze-drying and vacuum drying processes

- Park *et al.* 1997
- Freeze-thaw-tolerant yeast strains have higher levels of trehalose
- High tolerance to freezing during lag phase, low resistance during log phase
- Trehalose stabilizes the intracellular water structure and cell membranes under stress conditions
- Cells thawed at 0°C and room temperature did not differ in viability
- Ice can form intracellularly at high freezing rates
- External freezing precedes internal freezing, external freezing leads to dehydration and ice formation inside the cell

Strain variability

- Takagi *et al.* 1997
- Possible to “breed” freeze tolerance
- High gravity strains may tolerate freezing better

Why it works – slow freezing

- Komatsu *et al.* 1987
- Fast cooling of cells with liquid nitrogen results in damaging of all cellular membranes, including the nuclear one.
- Tanghe *et al.* 2002
- Rapid osmotically driven efflux of water during freezing reduces intracellular ice crystal formation and resulting cell damage
- Deletion of *AQY1* and *AQY2* renders yeast more sensitive to freezing, while overexpression improves freeze tolerance

Reviving

- Can pitch whole tube directly into a starter
 - Probably better to start with enriched media
- Can streak to plates and pick a single
 - Helps insure there is no contamination

Plates/Slants/Media

- Raines
- Agar plate: 1-4 weeks
- Agar slant: 0.5-2 years
- Yeast storage media and resuscitation formula

Dry malt extract	3.0 g
Glucose	10.0 g
Yeast nutrient	5.0 g
Agar	18.0 g
Water	to 1000 ml

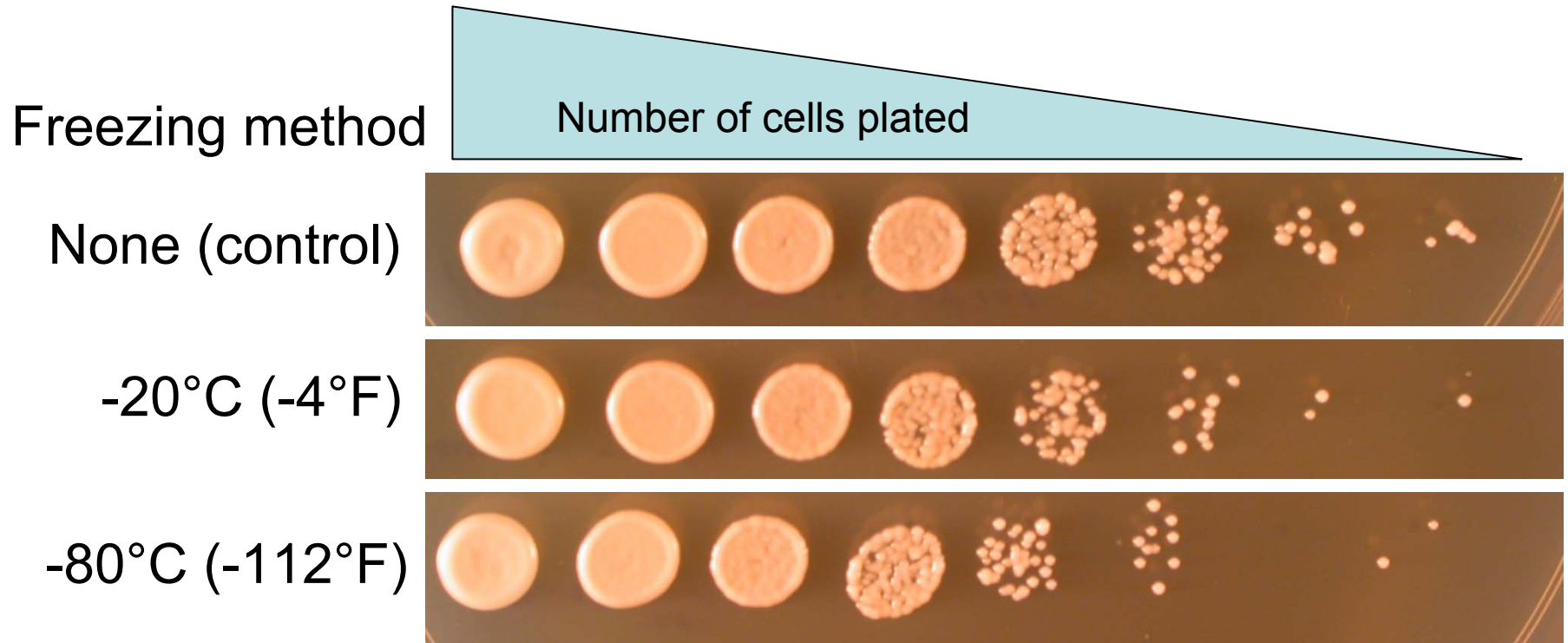
Starters

- Zainasheff
- S.G. between 1.020 and 1.040 or 5-10% malt extract
- 1/4 tsp yeast nutrient per 2 liters
- Boil 15 minutes, cool
- Increase volume by 5-10 times per step

My tests

- Freezing
 - Varied freezing methods and starting temp
 - Plate for viability
- Storage
 - Trial 1 ~ 16 weeks
 - Trial 2 ~ 8 weeks
 - Trial 3 ~ 8 weeks
 - Trial 4 ~ 6 weeks (ongoing)

Freezing Trials – Room Temp



1:5 serial dilutions, 4 μ l per spot, strain used is Wyeast 1056

- After freezing treatment, cells stored at -20°C (-4°F)
- Original concentration is ~74M CFU/ml
- Survival of cells is less than 20% via this treatment
- Slightly better in -20°C

Freezing Trials – Chilled First

Freezing method

Number of cells plated

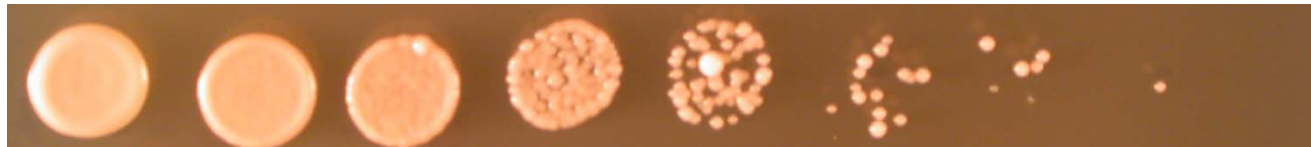
None (control)



-20°C (-4°F)



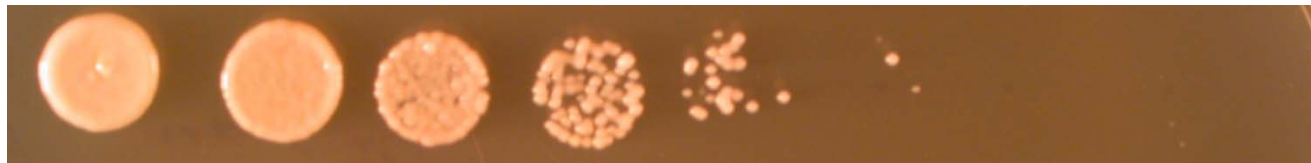
-80°C (-112°F)



Dry Ice (-109°F)



EtOH Bath (-109°F)



1:5 serial dilutions, 4 μ l per spot, strain used is Wyeast 1056

Freezing Trials – Chilled 3 hours

- After freezing treatment, cells stored at -20°C (-4°F)
- Original concentration is $\sim 76\text{M}$ CFU/ml
- Survival of cells $\sim 3\%$ on dry ice or in an ethanol bath
- Survival is $\sim 22\%$ when frozen at -80°C
- When frozen at -20°C directly, survival is $\sim 37\%$

Freezing Trials – Which is best?

- When freezing at -20°C , pre-chilling will more than double survival (36.6% vs. 16.5%)
- Affecting factors
 - Rate of cooling
 - Presence of cryoprotectants
 - Length of time stored at 4°C prior to freezing

Storage Trials

- Trial 1
 - Stored unprotected in freezer
 - NO viability after 16 weeks

Storage Trials

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- Trial 2
 - Why power is important

Storage Trials

- Trial 1
 - Stored unprotected in freezer
 - NO viability after 16 weeks
- Trial 2
 - Why power is important
- Trial 3
 - Why locks are good to have

Storage Trials – Number 4

- Room temp and refrigerated have highest viability after 4 weeks
- Samples lacking glycerol are dead
- Small difference between samples stored in cooler vs. not
- Difference between samples in cooler submerged in isopropanol or not

Other resources

- Zymurgy March/April 2007, Maribeth Raines
- http://www.maltosefalcons.com/tech/MB_Raines_Guide_to_Yeast_Culturing.php
- <http://www.wyeastlab.com/education/edyehist.htm>
- <http://www.wyeastlab.com/cbrew/cbyewash.htm>
- <http://www.wyeastlab.com/hbrew/hbyewash.htm>
- First Steps in Yeast Culture (Pierre Rajotte)

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Thanks for listening!

Feel free to email me at:

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This presentation and updates should (soon) be available at:

cascadebrewersclub.org/knowledge/yeast/