Micronutrients and yeast nutrition

High (excess) and low (limiting) concentrations inhibit yeast performance.

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Adjunct Professor
Phibro Fermentation Research
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Phibro Animal Health Corporation
A Global Manufacturer and Marketer of Animal Health & Nutrition and Performance Products

> $748 million in Global Sales

Supported by more than 1,200 employees worldwide

More than 450 product registrations in 65 countries across all continents

2,500+ customers in a wide variety of end-use markets

Experienced sales, marketing, and technical organization with more than 50 PhD’s, DVM’s, chemists, and technicians on staff

State-of-the-art laboratories and technical centers
Ethanol plant optimization
Ethanol plant optimization

Historical and traditional optimizations for yeast fermentations have centered around process conditions in propagators and fermentors.
Ethanol plant optimization

Historical and traditional optimizations for yeast fermentations have centered around process conditions in propagators and fermentors.

Standard yeast microbiology has been employed since the beginning of the ethanol industry and has served the industry well in scaling up to meet demand.
Standard yeast microbiological models cannot account for all of the process subtleties affecting yeast health at fuel ethanol plants.

Entire plant must be taken into account to fully understand all process conditions that affect yeast and achieve the next level of ethanol yield in existing plants.
Ethanol plant optimization (the next level)

Standard yeast microbiological models cannot account for all of the process subtleties affecting yeast health at fuel ethanol plants.

Entire plant must be taken into account to fully understand all process conditions that affect yeast and achieve the next level of ethanol yield in existing plants.
Nutrition and yeast
Nutrition and yeast

Yeast compositional analysis

Elemental composition¹

<table>
<thead>
<tr>
<th>Element</th>
<th>Yeasts (g/100 g dry matter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>47.0</td>
</tr>
<tr>
<td>H</td>
<td>6.0</td>
</tr>
<tr>
<td>O</td>
<td>33.2</td>
</tr>
<tr>
<td>N</td>
<td>7.5</td>
</tr>
<tr>
<td>K</td>
<td>3.5</td>
</tr>
<tr>
<td>P</td>
<td>1.2</td>
</tr>
<tr>
<td>S</td>
<td>0.3</td>
</tr>
<tr>
<td>Mg</td>
<td>0.15</td>
</tr>
<tr>
<td>Ca</td>
<td>0.09</td>
</tr>
<tr>
<td>Na</td>
<td>0.02</td>
</tr>
<tr>
<td>Zn</td>
<td>0.004</td>
</tr>
<tr>
<td>Fe</td>
<td>0.003</td>
</tr>
<tr>
<td>Cu</td>
<td>0.002</td>
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<tr>
<td>Mn</td>
<td>0.00004</td>
</tr>
<tr>
<td>Co</td>
<td>0.0005</td>
</tr>
<tr>
<td>Mo</td>
<td>0.000005</td>
</tr>
<tr>
<td>Cl</td>
<td>0.004</td>
</tr>
<tr>
<td>Li</td>
<td>0.00005</td>
</tr>
<tr>
<td>Pb</td>
<td>0.0001</td>
</tr>
<tr>
<td>As</td>
<td>0.0001</td>
</tr>
<tr>
<td>Si</td>
<td>—</td>
</tr>
<tr>
<td>Sr</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>—</td>
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Composition of the cell²

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<tr>
<td>Ash</td>
<td>45–75</td>
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</tr>
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<td>Nucleic acid (DNA and RNA)</td>
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</tr>
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<td>Lipids</td>
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</tr>
<tr>
<td>Phosphorus</td>
<td>10–19</td>
<td></td>
</tr>
<tr>
<td>Sulfur</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>20–21</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>0.12–0.3</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>0.6–0.75</td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>0.02–0.03</td>
<td>0.1</td>
</tr>
<tr>
<td>Magnesium</td>
<td>1.3–1.65</td>
<td>2.3</td>
</tr>
<tr>
<td>Cobalt</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Zinc</td>
<td>0.170–0.197</td>
<td></td>
</tr>
<tr>
<td>Chromium</td>
<td>0.0005–0.0025</td>
<td></td>
</tr>
<tr>
<td>Nickel</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Tin</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Molybdenum</td>
<td>0.00004</td>
<td></td>
</tr>
<tr>
<td>Lithium</td>
<td>0.000017</td>
<td></td>
</tr>
<tr>
<td>Vanadium</td>
<td>0.00004</td>
<td></td>
</tr>
<tr>
<td>Selenium</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Pantothenate (Coenzyme A)</td>
<td>0.085–0.10</td>
<td>0.110–0.120</td>
</tr>
<tr>
<td>Choline (membranes)</td>
<td>2.71–4.00</td>
<td>3.80–4.55</td>
</tr>
<tr>
<td>Thiamin (Vit B1)</td>
<td>0.099–0.150</td>
<td>0.092–0.150</td>
</tr>
<tr>
<td>Riboflavin (Vit B2)</td>
<td>0.150–0.100</td>
<td>0.045–0.045</td>
</tr>
<tr>
<td>Nicotinic acid/niacin (NAD)</td>
<td>0.20–0.585</td>
<td>0.450</td>
</tr>
<tr>
<td>Pyridoxine (Vit B6)</td>
<td>0.020–0.040</td>
<td>0.043–0.050</td>
</tr>
<tr>
<td>Biotin (Biotin)</td>
<td>0.0006–0.0013</td>
<td></td>
</tr>
<tr>
<td>p-aminobenzoic acid (follic)</td>
<td>0.160</td>
<td></td>
</tr>
<tr>
<td>Inositol (phospholipids)</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Folic acid ([1-C transfer)</td>
<td>0.013–0.015</td>
<td>0.010</td>
</tr>
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¹ Elemental composition
² Composition of the cell
Elemental composition

Composition of the cell

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<td></td>
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Composition within yeast cell is only meant to be a guideline - e.g. “Hoarding”, exclusion from cell, metabolic degradation, growth conditions, and other microbiological factors all detract from amounts within cell.

Optimal levels for some ions/minerals to stimulate fermentation are higher than indicated on yeast cell compositional analysis.
Nutrition and yeast

Biological availability of minerals

Biological availability not always a simple as assessing total amount in mash. Complicating inorganic ion stimulation/inhibition/nutrition on yeast:
Nutrition and yeast

Biological availability of minerals

Biological availability not always a simple as assessing total amount in mash. Complicating inorganic ion stimulation/inhibition/nutrition on yeast:

1. Availability of an ion for the yeast may be dependent on the concentration of other ions present. (e.g. uptake of Mg$^{2+}$ influenced by Ca$^{2+}$)

2. Any metal ion may be present as several different species (e.g. different oxidation states, complexes, protein complexation) - only some of which are bioavailable for the yeast.

3. Availability of some ions depend on pH and on any chelating agents in the mash. (e.g. yeast can produce succinic, malic, and citric acids – all metal ion chelators)

4. Rate of equilibrium of chelating agent-ion complex.

5. Chemical interactions between different ions. (e.g. SO$_2^-$, ClO$_2^-$ oxidation of ions)

6. Conditions used to process mash. (e.g. higher temperatures, pH can oxidize minerals)

7. Preferential active uptake of ions by yeast. (e.g. Mg, Co and Zn > Mn > Ni > Ca > Sr)
Nutrition and yeast
Essential/nonessential for nutrition

Is a mineral essential/nonessential for yeast?

Increasing concentration of mineral exhibits toxic effects on yeast (symptoms similar to Non-Essential mineral)

Some effects on yeast:
- Slower growth rate
- Slower fermentation rate
- Cell membrane oxidation
- Yeast enzyme inactivation
- Permanent changes in yeast DNA

Essential
Minerals: e.g. nitrogen, potassium, calcium, magnesium, phosphorus, sulfur

Non Essential
Minerals: e.g. sodium, mercury, lead, chlorine, bromine, iodine
Nutrition and yeast

Essential/nonessential for nutrition

Is a mineral essential/nonessential for yeast?

In each pair of corresponding zones, toxic and beneficial microbiological effects show the same degree of effect.

Based on this fact, it is extremely difficult to know for a particular concentration what part of each curve a particular mineral is part of.

Yeast foods typically add multiple minerals to stimulate yeast
  - Are each one optimized?
  - Additive effects?
  - Synergistic effects?
  - Detrimental effects?
## Nutrition and yeast

Minerals needed for enzymes

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Enzyme cofactor/ Effect on yeast</th>
<th>Structural component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium</td>
<td>pyruvate kinase, aldehyde dehydrogenase, aldolase, membrane ATPase, pH buffering, ergosterol biosynthesis regulation</td>
<td>Ribonucleic acids</td>
</tr>
<tr>
<td>Sodium</td>
<td>Not required</td>
<td>Proteins (~80%), nucleic acids, coenzymes</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>(as NH₄⁺) phosphofructokinase, oxidoreductase, aldolase</td>
<td>Proteins (~80%), nucleic acids, coenzymes</td>
</tr>
<tr>
<td>Calcium</td>
<td>pyruvate dehydrogenase, phosphatase, proteinase, transketolase, required for flocculation and budding</td>
<td>Cell wall proteins, stabilizes cell membrane</td>
</tr>
<tr>
<td>Magnesium</td>
<td>hexokinase, phosphofructokinase, phosphoglycerate kinase, enolase, pyruvate decarboxylase, fumerase, pyruvate kinase, membrane ATPase, pyruvate carboxylase, inorganic pyrophosphatase, ATP-sulfurylase</td>
<td>Ribosomes, cell membranes, nucleic acids, phospholipids, polyphosphates</td>
</tr>
<tr>
<td>Sulfur</td>
<td>(as SO₄²⁻) phosphoglycerate kinase</td>
<td>Proteins, free amino acids</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>(as PO₄³⁻); ATP, other energy transferring compounds</td>
<td>Nucleic acids, phospholipids, cell wall polymers, phosphate esters</td>
</tr>
</tbody>
</table>

### Glucose

Glucose is a nutrient for yeast and is used in the fermentation process to produce ethanol.

### Ethanol

Ethanol is a product of the fermentation process and is formed when yeast metabolizes glucose.
Nutrition and yeast

Sodium/Potassium

Corn (32% mash): 141 ppm
Required (min) by yeast: – ppm
Nutrition and yeast

Sodium/Potassium

Sodium not required by yeast

Negative effects on yeast:

Inhibits K⁺ uptake (both K⁺ and Na⁺ compete for same transport system)

Inhibits membrane ATPase (used by yeast to export H⁺ ions – crucial for yeast growth)

Inhibits glucose uptake, amino acid uptake

Solvent effect on proteins

Inhibits glycolytic enzymes within yeast: hexokinase, aldolase, triosephosphate isomerase

Inhibition on yeast reported from 500 ppm up to 5000 ppm (as individual ion). Most likely ion in excess amounts in recycled backset
Nutrition and yeast

Sulfur

Required by yeast for amino acids and proteins

No positive effects on yeast in excess

Negative effects on yeast:

Least toxic forms: Amino acids, SO$_4^{2-}$

More toxic forms: Sodium Sulfite/Bisulfite Na$_2$SO$_3$/NaHSO$_3$ ~ 100 ppm
Sulfamic acid H$_3$NSO$_3$
Magnesium/Zinc sulfate MgSO$_4$/ZnSO$_4$
Sulfuric acid H$_2$SO$_4$

SO$_4^{2-}$ causes uptake of 3 H$^+$ and expels K$^+$. Disrupts membrane pH gradient. Uptake is higher at lower pH

Excess glycerol and/or fusel production by yeast. Oxidation of membrane (yeast lysis), oxidation of enzymes within yeast

Corn (32% mash): 235 ppm
Required (min) by yeast: 32 ppm
Nutrition and yeast

Calcium

Positive effects on yeast

- Required by yeast for a few enzymes: pyruvate dehydrogenase, phosphatase, transketolase
- Required for budding, cell wall proteins
- Stabilizes cell membranes
- Needed in very small amounts. Yeast cell actively expels calcium.

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Medium</th>
<th>Mg (mg/L)</th>
<th>Ca (mg/L)</th>
<th>Mg/Ca</th>
<th>Final Ethanol (% v/v)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>White wine must</td>
<td>67</td>
<td>136</td>
<td>0.5</td>
<td>7.1</td>
<td>0 (control)</td>
</tr>
<tr>
<td>S. cerevisiae DCLM</td>
<td>1.217</td>
<td>114</td>
<td>10.7</td>
<td>4.8</td>
<td>0.25</td>
<td>+1.2</td>
</tr>
<tr>
<td>S. cerevisiae NCYC 1109</td>
<td>Corn molasses</td>
<td>100</td>
<td>450</td>
<td>0.22</td>
<td>5.6</td>
<td>0 (control)</td>
</tr>
<tr>
<td>S. cerevisiae NCYC 1109</td>
<td>Malt wort</td>
<td>190</td>
<td>450</td>
<td>0.42</td>
<td>6.6</td>
<td>+1.2</td>
</tr>
<tr>
<td>S. cerevisiae NCYC 1109</td>
<td>(OG 1062)</td>
<td>257</td>
<td>710</td>
<td>0.36</td>
<td>3.8</td>
<td>0 (control)</td>
</tr>
<tr>
<td>S. cerevisiae DCLM</td>
<td>Chemically defined</td>
<td>265</td>
<td>1,675</td>
<td>0.16</td>
<td>3.7</td>
<td>0 (control)</td>
</tr>
<tr>
<td>S. cerevisiae DCLM</td>
<td>Chemically defined</td>
<td>280</td>
<td>2,100</td>
<td>0.13</td>
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Corn (32% mash): 141 ppm
Required (min) by yeast: 10 ppm
Nutrition and yeast

Calcium

### Negative effects on yeast

Excess amounts inhibitory for yeast

Strong competitor to Mg\(^{2+}\).

10:1 ratio of Ca\(^{2+}\)/Mg\(^{2+}\) prevents yeast growth

3:1 ratio increases yeast lag phase

Antagonizes many Mg\(^{2+}\) dependent functions of yeast growth thru competition

Effects of Ca\(^{2+}\) mainly exerted outside the cell and can influence pH

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**Influence of Magnesium and Calcium Variability on Yeast Fermentation Performance**

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<td>DBV 2106</td>
<td></td>
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<td>114</td>
<td>10.7</td>
<td>8.7</td>
<td>+1.6</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Canic malolase</td>
<td>47</td>
<td>768</td>
<td>0.06</td>
<td>4.8</td>
<td>-2.3</td>
</tr>
<tr>
<td>DCLM</td>
<td></td>
<td>1100</td>
<td>1100</td>
<td>0.22</td>
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Corn (32% mash): 141 ppm

Required (min) by yeast: 10 ppm
Nutrition and yeast

Calcium

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Corn (32% mash): 141 ppm
Required (min) by yeast: 10 ppm
Nutrition and yeast

Zinc

Positive roles for Zinc

- Essential mineral (structural) for ethanol production enzymes: alcohol dehydrogenase, aldolase, acetaldehyde dehydrogenase
- Stimulates uptake of maltose and maltotriose
- Increased tolerances to acetic acid, ethanol tolerance, heat shock, osmotic shock, oxidative stress, salt stress (halotolerance)
- Cellulose: Increased tolerance to hydroxymethylfurfural (HMF) (non-enzymatic hydrolysis)
- Regulation of yeast cell cycle growth

Corn (32% mash): 6.58 ppm
Required (min) by yeast: 0.98 ppm

Ethanol yield increase up to 26.5 ppm
Maximum yield increase at 2.5 ppm
Nutrition and yeast

**Zinc**

Ethanol yield increase up to 26.5 ppm
Maximum yield increase at 2.5 ppm

**Negative roles for Zinc**

At higher concentrations:

- Activation of internal degradative enzymes,
- Suppression of metabolic pathways that aid in detoxifying yeast
- Disruption of membrane structure, leakage of intracellular components out the cell
- Activation of self autolysis (lysis)

Corn (32% mash): 6.58 ppm
Required (min) by yeast: 0.98 ppm
Nutrition and yeast

Magnesium

Positive roles for Magnesium

Known for decades as a stimulator for yeast for fermentation.

Second highest concentration within yeast next to K⁺

Essential for proper enzyme function (over 300) within yeast

Plays an essential role in control of cell growth, cell division, and size.

Yeast actively accumulates Mg²⁺ (spends energy to accumulate)
Nutrition and yeast

Magnesium

**Negative roles for Magnesium**

Growth rate limited at 0-650 µM Mg\(^{2+}\) (~42.5 ppm)

Growth rate inhibited at 25,000 ppm (~1M). (no practical yeast inhibition at an ethanol plant)
Nutrition and yeast

Mineral interactions

Absolute effects of each ion on yeast is known and researched extensively.

Can list beneficial/detrimental amounts of each individual anion/cation for yeast.

Nearly impossible to predict overall effect on yeast with multiple cations/anions present, with different concentrations of each, and with higher overall levels of addition of all species.

A few examples (many others undergoing research):
Nutrition and yeast

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A few examples (many others undergoing research):

- Reduces uptake of Mg
- Decreases yeast growth rate
- Decreases cell division

Yeast growth rate
Cell division

Reduced uptake of Mg
Decreased yeast growth rate
Decreased cell division
Nutrition and yeast

Mineral interactions

High concentrations of
Zn$^{2+}$ or Cu$^{2+}$ or Cd$^{2+}$

Damaged yeast membrane
Yeast cell lysis
Leakage of K$^+$

High uptake

Lower uptake of all

Nutrition and yeast

Mineral interactions

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Damaged yeast membrane
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Leakage of K$^+$

High uptake

Lower uptake of all
Nutrition and yeast
Mineral interactions

- Reduction in cell growth
- Increase lag phase

High Zn$^{2+}$

- Uptake of toxic ions
- Mg$^{2+}$
- 3 Ca$^{2+}$ / Mg$^{2+}$
- 10 Ca$^{2+}$ / Mg$^{2+}$

- Sn$^{2+}$, Hg$^{2+}$, SO$_4^{2-}$, Co$^{2+}$, Mn$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, Cu$^{2+}$, Cr$^{2+}$
Setting a limit of “x” for e.g. sodium, chloride, etc for fermentation is a moving target. Recycle/water composition constantly changes and along with them the negative/positive effects on yeast.

Interaction of ions with yeast are extremely complicated. No one has yet determined all permutations

Yeast has a preferential order of uptake of minerals

Adding excess amounts a particular ion can have multiple positive and negative unplanned effects on the yeast.

Reduction of sodium, $\text{Na}_2\text{SO}_3/\text{NaHSO}_3$, Cl$^-$, general guidelines to limit concurrent negative effects with other ions.
Yeast foods may aid in yeast growth and fermentation, but care needs to be taken on overuse and formulation.

Impossible to “point” to a compositional analysis of mash/recycle streams and say Mineral “X” is a problem for the yeast. All minerals can have positive/negative effects.
GMO Yeast

Commercially in the last 5 years there has been an explosion of GMO yeast targeted for the fuel ethanol industry.

Very exciting time to be involved with yeast in fuel ethanol industry. Plenty of innovation coming from research labs.

- Amylase expression (8 different GMO options)
- Protease expression (5 different GMO options)
- Glycerol reduction (11 different GMO options)
- Ethanol yield increase (3 different GMO options)
- Membrane/ cell wall stabilization (0 different GMO options)
Glucoamylase GMO Yeast
Glucoamylase GMO Yeast

Starches
- Corn
- Milo
- Wheat
- Peas
- Potato
- Rice
- Tubers
- Barley

Conversion
- Alpha Amylase
- Glucoamylase

Glucose (Dextrose)

Traditional glucoamylase addition strategy

- Batch addition
  - 1st addition (~ 3% fill, before yeast addition)
  - 2nd addition (50% fill)

- Semi-continuous addition
  - 1st addition (prior to yeast addition)
  - Continuous addition

Conversion
- Mash = Corn, starch
- Malt = Beer
- Wort = Molasses
- Must = Wine
Glucoamylase GMO Yeast


Immobilized on cell surface  Secreted
Early work demonstrating glucoamylase gene added to *S. cerevisiae*

Both immobilized and secreted forms of glucoamylase tested

Laid the foundation for further improvements in stability of GA expression, GA activity, higher ethanol content
Glucoamylase GMO Yeast


Advantages for the ethanol plant
1. Reduction of glucoamylase addition
2. Fewer fermentor errors (operators)

Advantages for the yeast
1. Glycoamylase produced by yeast when required for growth
2. Potential reduction in bacterial contamination as excess glucose not present.
3. Reduced possibility of osmotic shock due to overconversion of glucose

Disadvantages for yeast
1. Yeast growth MUST occur for glucoamylase to be produced
2. Expression of glucoamylase must occur before exponential growth of yeast
Glucoamylase GMO Yeast

Timing of glucoamylase expression
Glucoamylase GMO Yeast
Timing of glucoamylase expression

Maximum growth rate of yeast between 6 and 24 hours into fermentation

Highest demand for carbohydrate nutrition
Glucoamylase GMO Yeast

Timing of glucoamylase expression

With traditional glucoamylase addition, Glucose is available to the yeast as soon as it is added.

\[ y = Ae^{-\left(\frac{\mu_{\text{max}} e^{(\lambda - t)} - 1}{1 + \lambda}\right)} \]
Glucoamylase GMO Yeast

Timing of glucoamylase expression

With self production of glucoamylase, yeast must be healthy enough to produce GA, produce it prior to exponential growth, and produce enough of it to satisfy fermentation kinetics.

\[ y = Ae^{-\frac{\mu_{\text{max}}(\lambda t - 1)}{A} + 1} \]
Glucoamylase GMO Yeast

On the horizon for GMO yeast to complement glucoamylase substrate conversion:

Most of these have already been successfully constructed and tested at research facilities:

Full glucoamylase replacement (Currently some glucoamylase is still needed during fermentor fill)

Amylases (endo α1-4 starch hydrolysis)

Pullilonases (endo α1-3, endo α1-3, enco α1-6 starch hydrolysis)

Cellulases (endo/exo β1-4, endo β1-3, endo β1-6 cellulose hydrolysis)
Protease GMO Yeast
# Protease GMO Yeast

**Why add protease?**

1. Aids in breakdown of corn kernel

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**Distribution of major components in corn and some corn processing by-products**

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<thead>
<tr>
<th>Component</th>
<th>Whole Kernel (%)</th>
<th>Dry weight of components (%)</th>
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</tr>
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<td>7.8</td>
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</tr>
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</tbody>
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* By difference. Includes fiber, nonprotein nitrogen, pentosans, phytic acid, soluble sugars, xanthophylls.
** Also includes glycerol, organic acids and other byproducts of ethanol fermentation.
2. Increased Oil yield

Corn oil yield increases up to 11% over normal operation.

Energy input reductions
Ethanol yield increases

### Distribution of major components in corn and some corn processing by-products

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* By difference. Includes fiber, nonprotein nitrogen, pentosans, phytic acid, soluble sugars, xanthophylls.

** Also includes glycerol, organic acids and other byproducts of ethanol fermentation.
3. Provides additional FAN (Free Amino Nitrogen) for yeast nutrition

Corn protein

\[ \text{H}_2\text{N} \quad (\quad)_x \quad \text{COOH} \quad x = 0 \text{ to } 200,000 \]
How much FAN Nitrogen do we need for fermentation?

Why do we target 300 ppm FAN?

- Anaerobically grown yeast 37% to 42% protein.
- Yeast is ~6% Nitrogen.
- Cell density peak in fermentation ~ $250 \times 10^6$ cells/mL.
- $1 \text{ g yeast} = 5 \times 10^{10}$ cells
- $250 \times 10^6 / 5 \times 10^{10} = 0.005 = 0.5\%$ yeast mass
- $0.005 \times 0.06 = 0.0003$ Nitrogen
- $0.0003 \times 1,000,000 = 300 \text{ ppm FAN Nitrogen needed (Minimum)}$

Free Amino Nitrogen or FAN

Amino nitrogen-containing chemical that the yeast can biologically use

FAN is Different than %N (composition)

Not all N containing chemicals can be used by yeast

- e.g. Proteins in mash cannot be used directly by yeast
Protease GMO Yeast

Potential issues in using protease

- Timing of protease production (same as for amylase inclusion)
- Reduction in protein quality/quantity in DDGS
- Fusel production
Protease GMO Yeast
Reduction in protein quality/quantity in DDGS

Is there a risk to DDGS protein content or protein composition?

Calculations

- Fermentor volume (gal) 750,000
- Fermentor volume (L) 2,838,750
- Dry urea dose (lbs) 4000
- Dry urea dose (kg) 1,814
- Calculated FAN content for yeast (ppm) 341
- Molecular weight of urea (g/mol) 60.06
- Average molecular weight amino acid (g/mol) 131.92
- Amino acid mass equivalent to urea dose (lbs) 8,786
- Amino acid mass equivalent to urea dose (kg) 3,985
- Protein mass required (lbs) 8,786
- Protein mass required (kg) 3,985
  - Assumption: 1 protein molecule
- Total protein amount in fermentor (lbs) 220,316
- Total protein amount in fermentor (kg) 99,934
Protease GMO Yeast
Reduction in protein quality/quantity in DDGS

Is there a risk to DDGS protein content or protein composition?

Calculations (cont)
Protein mass required (lbs) 8,786
Protein mass required (kg) 3,985
   Assumption: 1 protein molecule
Total protein amount in fermentor (lbs) 220,316
Total protein amount in fermentor (kg) 99,934

Corn protein required to be hydrolyzed (%) 3.99

Typical fermentor has enough protein that (when hydrolyzed) can supply all FAN nutritional requirements of yeast.
Is there a risk to DDGS protein content or protein composition?

Not all of the amino acids that the yeast accumulates from the mash is funneled into proteins (misconception in industry)

Once the fermentable carbohydrate in the fermentor is depleted (~50 hours), the yeast will have no choice but to:

1. Use amino acids for energy production
2. Use amino acids as a C source for metabolism
3. Produce more fusels as a byproduct of energy production
Are there any differences for the yeast in fermenting amino acids?

**Ehrlich pathway**

- **Amino Acids**
- **α keto acid**
  - 1. Transamination
  - 2. Decarboxylation
  - Reduction
  - Oxidation

- **‘fusel aldehyde’**
- **‘fusel acid’**
  - Export
  - ATP
- **‘fusel alcohol’**
  - Diffusion
  - ATP

Production of α keto acid point of "no return" for yeast and fusels

Flavor/aroma/volatile compounds
Protease GMO Yeast

Advantages for the ethanol plant
1. Reduction of protease addition
2. Fewer fermentor errors (operators)
3. (Production advantages of using proteases)

Advantages for the yeast
1. Protease produced by yeast when required for growth
2. Potential reduction in bacterial contamination as excess glucose not present.
3. FAN produced with yeast demand

Disadvantages for yeast
1. Yeast growth MUST occur for protease expression
2. Reduction in DDGS protein quality
3. Fusel production
Glycerol reduction GMO Yeast
Glycerol reduction GMO Yeast

Yeast production of glycerol
Glycerol reduction GMO Yeast

Yeast production of glycerol
Glycerol reduction GMO Yeast

Yeast production of glycerol
Glycerol reduction GMO Yeast

Yeast production of glycerol

To balance NAD/NADH redox yeast can:

1. Increase glycerol production
Glycerol reduction GMO Yeast

Yeast production of glycerol

To balance NAD/NADH redox yeast can:
1. Increase glycerol production
2. Increase fusel production
Glycerol reduction GMO Yeast

Yeast production of glycerol

To balance NAD/NADH redox yeast can:
1. Increase glycerol production
2. Increase fusel production
3. GMO yeast
GMO reduction of glycerol:

GMO deletion (X) of enzyme glycerol-3-phosphate dehydrogenase and let yeast sort out regenerating NAD somehow.
GMO deletion (X) of enzyme glycerol-3-phosphate dehydrogenase

Unsuccessful first GMO attempt to remove glycerol

Yeast requires glycerol for proper membrane stabilization against common yeast stresses: osmotic, salt, temperature, lactic acid, acetic acid, fusels, and other stressors

Produced yeast had no glycerol production, but had no stress tolerance and could not balance redox

Cannot fully remove glycerol
GMO insertion of enzymes from bacteria:

1. pyruvate formate lyase (PFL)
   
   \[ \text{pyruvate} \leftrightarrow \text{formate} + \text{acetyl CoA} \]

2. PFL activase/deactivase enzymes
   
   Regulatory enzymes: Turns PFL “on” or “off”

3. acetaldehyde dehydrogenase
   
   \[ \text{acetyl CoA} + \text{NADH} + \text{H}^+ \leftrightarrow \text{acetaldehyde} + \text{NAD}^+ + \text{CoA} \]
Glycerol reduction GMO Yeast

Insertion of enzymes

Assuming PFL activase present and PFL “on”

\[
\text{puruvate} \leftrightarrow \text{formate} + \text{acetyl CoA}
\]

\[
\text{acetyl CoA} + \text{NADH} + \text{H}^+ \leftrightarrow \text{acetaldehyde} + \text{NAD}^+ \text{H}^+ + \text{CoA}
\]
Glycerol reduction GMO Yeast

Insertion of enzymes

Assuming PFL activase present and PFL “on”

\[ \text{Puruvate} \leftrightarrow \text{Formate} + \text{Acetyl CoA} \]

Extremely complicated genetic manipulation to get enzyme systems into yeast, expressed at high levels, stable expression, proper regulation, and balance in yeast stress response.

Advantages for yeast:

- Redox balance better maintained within yeast
- Production of (some) glycerol maintained for yeast cell membrane stability
Glycerol reduction GMO Yeast

Insertion of enzymes

Disadvantages for yeast:

1. Loss of formate (and ethanol production) – loss of carbon

\[
\text{formate} + \text{NAD}^+ \rightarrow \text{CO}_2 + \text{NADH}
\]

\text{formate reductase}

2. Toxicity of formate (formic acid) on yeast

Assuming PFL activase present and PFL “on”

\[
\text{purovate} \leftrightarrow \text{formate} + \text{acetyl CoA}
\]

\[
\text{acetyl CoA} + \text{NADH} + \text{H}^+ \leftrightarrow \text{acetaldehyde} + \text{NAD}^+ \text{H}^+ + \text{CoA}
\]
Glycerol reduction GMO Yeast
Toxicity of formic acid (fatty acid inhibition on yeast)

Undissociated fatty acid

\[ {\text{Undissociated fatty acid}} \]

\[ \text{pH decrease} \]

\[ \text{pH increase} \]

Dissociated fatty acid

\[ \text{Inhibition on yeast} \]

\[ \text{Maximum delta in undissociated fatty acids occurs over fermentation/propagation pH range} \]

% Undissociated fatty acid various pH values

(Bayrock, 2017)
Glycerol reduction GMO Yeast
Toxicity of formic acid (fatty acid inhibition on yeast)

10 mM ~ 460 mg/L (ppm) ~ 0.046 %w/v

Ethanol concentration and yield summary from Saccharomyces spp fermentation of glucose in the presence of formic acid. Data are the biclone experiments and standard deviation.

(Oshoma et al, 2015)

Fig 3. Growth profile (A and C) and percentage tolerance (B and D) of Saccharomyces arboricola 2.3317, 2.3318 and 2.3319 and Saccharomyces cerevisiae NCYC2592 in the presence of formic acid (0–50 mM) using YPD medium. Values are the mean of three experiments and vertical error bars represent standard deviation.

(Oshoma et al, 2015)
Glycerol reduction GMO Yeast
Toxicity of formic acid (fatty acid inhibition on yeast)

Active research ongoing to find means to lessen toxic effects of formic acid

1. Reduce concentration of formate in cellulose hydrolysates
2. Addition of amino acid proline partially counteracts effects of formic acid

(Oshoma et al, 2015)
Thank you for your attention!

Questions?